

# **Aromatic Alcohol Oxidase, Aldehyde Oxidase and Mannitol Oxidase in Terrestrial Gastropods.**

**ANDREW LARGE B.Sc. (Hons)**

A thesis submitted in partial fulfilment of the requirements of  
the University of Wolverhampton for the degree of  
Doctor of Philosophy.

UNIVERSITY OF WOLVERHAMPTON LIBRARY	
Acc No. <b>887761</b>	CLASS <input type="checkbox"/>
CONTROL <b>95168833</b>	THESIS COLLECTION
DATE 5 FEB 1995	SITE <b>RS</b>

February 1995

# CONTENTS

<b>Contents</b>	<b>i</b>
<b>Publications Arising From This Research</b>	<b>v</b>
<b>Acknowledgments</b>	<b>vi</b>
<b>Abbreviations</b>	<b>vii</b>
<b>Abstract</b>	<b>1</b>
<b>1 Introduction</b>	<b>2</b>
1.1 General Introduction	2
1.1.1. History of the Study	2
1.1.2. Aims and Scope of the Investigation	3
1.2. The Generation and Detoxification of Hydrogen Peroxide and Other Reactive Oxygen Species	5
1.2.1. Subcellular Sites of Hydrogen Peroxide Generation	5
1.2.1.1. Mitochondria	5
1.2.1.2. Peroxisomes	7
1.2.1.3. Endoplasmic Reticulum	7
1.2.1.4. Cytosol	8
1.2.1.5. Other Organelles	8
1.2.2. The Detoxification of Hydrogen Peroxide and Other Related Oxygen Species	9
1.2.2.1. Toxicity of Reactive Oxygen Species	9
1.2.2.2. Antioxidant Enzymes	9
1.2.2.3. Other Cellular Defences Against Reactive Oxygen Species	11
1.3. Alcohol Oxidising Enzymes	12
1.3.1. Catalase	13
1.3.2. Microsomal Ethanol Oxidising System	13
1.3.3. NAD(P) <sup>+</sup> -linked Dehydrogenases	14
1.3.4. NAD(P) <sup>+</sup> -independent Alcohol Dehydrogenases	16
1.3.5. Alcohol Oxidases	17

1.4.	Aldehyde Oxidation	21
1.4.1.	NAD(P) <sup>+</sup> -dependent Aldehyde Dehydrogenases	21
1.4.2.	NAD(P) <sup>+</sup> -independent Aldehyde Dehydrogenases	23
1.4.3.	Aldehyde Oxidases	24
1.5.	Sugar Alcohol Oxidation	27
1.5.1.	NAD(P) <sup>+</sup> -dependent Sugar Alcohol Dehydrogenases	28
1.5.1.1.	Hexitol Dehydrogenases	28
1.5.1.2.	Hexitol-phosphate Dehydrogenases	29
1.5.1.3.	Pentitol Dehydrogenases	30
1.5.2.	NAD(P) <sup>+</sup> -independent Sugar Alcohol Dehydrogenases	31
1.5.3.	Sugar Alcohol Oxidases	31
2.	<b>Materials and Methods</b>	34
2.1.	Animals	34
2.2.	Subcellular Fractionation	35
2.2.1.	Preparation of Homogenates	35
2.2.2.	Sucrose Density Gradient Centrifugation Methods	35
2.2.2.1.	Isopycnic Gradient Method Using the BXIV Rotor with the B29 Insert - Method for <i>Arion ater</i>	35
2.2.2.2.	Isopycnic Gradient Method Using the BXIV Rotor with the B29 Insert - Method for <i>Achatina fulica</i>	36
2.2.2.3.	Rate Dependent Sedimentation in a Sucrose Gradient Using the BXIV Rotor - Method for <i>Arion ater</i>	37
2.2.2.4.	Dual Gradient Method for the Enrichment of Mannitol Oxidase Bearing Membranes - <i>Arion ater</i>	37
2.2.2.5.	Isopycnic Gradient Method Using the Beckman Ti-50 Vertical Rotor	38
2.2.2.6.	Rate Dependent Sedimentation Using the MSE 6 x 38ml Swing-Bucket Rotor	39

2.2.3.	Analysis of Fractions	40
2.2.3.1.	Sucrose	40
2.2.3.2.	Protein	40
2.2.3.3.	Mannitol Oxidase	41
2.2.3.4.	Aromatic Alcohol Oxidase	43
2.2.3.5.	Semicarbazide-Sensitive Amine Oxidase	44
2.2.3.6.	Succinate Dehydrogenase	44
2.2.3.7.	Catalase	45
2.2.3.8.	Alkaline Phosphatase	46
2.2.3.9.	Aryl Sulphatase	46
2.2.3.10.	$\beta$ -Glucuronidase	47
2.2.3.11.	Esterase	47
2.2.3.12.	Acid Phosphatase	48
2.2.3.13.	NADPH-Cytochrome c Reductase	49
2.2.3.14.	Thiamine Pyrophosphatase	49
2.2.4.	Presentation of Fractionation Results	51
2.2.5.	Electron Microscopy	51
2.2.6.	S.D.S.-P.A.G.E. Analysis	52
2.3.	Sample Preparation and Characterisation of Catalytic Activities	53
2.3.1.	Preparation of Samples for Enzyme Analysis	53
2.3.1.1.	Aromatic Alcohol Oxidase	53
2.3.1.2.	Aldehyde Oxidase	56
2.3.1.3.	Mannitol Oxidase	56
2.3.2.	Assay Methods	57
2.3.2.1.	Aromatic Alcohol Oxidase	57
2.3.2.2.	Aldehyde Oxidase	64
2.3.2.3.	Mannitol Oxidase	65
2.3.2.4.	Protein Assay	65
2.3.3.	Cellulose Acetate Electrophoresis	66
2.3.4.	Estimation of Apparent $K_m$ Values	67
3.	Results	68
3.1.	Subcellular Fractionation Studies	68
3.1.1.	Fractionation of <i>Arion ater</i> Digestive Gland	68
3.1.1.1.	Density-dependent Banding	68
3.1.1.2.	Rate-dependent Banding	71
3.1.1.3.	Dual Gradient Method for the Isolation of Mannitol Oxidase Carrying Structures and their Identification	72
3.1.2.	Fractionation of <i>Achatina fulica</i> Digestive Gland P.N.S.	74
3.1.2.1.	Density-dependent Banding	74



3.1.3.	Comparative Analytical Fractionation of Digestive Gland P.N.S. Derived from Several Species of Gastropod Mollusc	76
3.1.3.1.	Density-dependent Banding	76
3.1.3.1.	Rate-dependent Banding	78
3.2.	Characterisation of Catalytic Activities	80
3.2.1.	Aromatic Alcohol Oxidase	80
3.2.1.1.	Aromatic Alcohol Oxidase Activity in <i>Arion ater</i>	80
3.2.1.2.	Comparison of Aromatic Alcohol Oxidase Activity in Three Species of Terrestrial Gastropod Mollusc	83
3.2.1.3.	Alcohol Oxidase Activity in <i>Achatina fulica</i>	85
3.2.2.	Aldehyde Oxidase	88
3.2.2.1.	Comparison of Aldehyde Oxidase Activity in Three Species of Gastropod Mollusc and Mouse Liver	88
3.2.2.2.	Aldehyde Oxidase Activity in the Tissues of <i>Achatina fulica</i>	90
3.2.3.	Mannitol Oxidase	92
3.2.3.1.	Mannitol Oxidase Activity in Four Species of Gastropod Mollusc	92
4.	Discussion	93
4.1.	The Subcellular Fractionation of Gastropod Digestive Gland	93
4.1.1.	The Mannosome	93
4.1.2.	The Subcellular Localisation of Aromatic Alcohol Oxidase	102
4.2.	Characterisation of Catalytic Activities	103
4.2.1.	Aromatic Alcohol Oxidase	103
4.2.1.1.	Aromatic Alcohol Oxidase Activity in <i>Arion ater</i> and Other Temperate Gastropod Species	103
4.2.1.2.	Alcohol Oxidase Activity in <i>Achatina fulica</i>	108
4.2.2.	Aldehyde Oxidase	110
4.2.3.	Mannitol Oxidase	112
	Bibliography	114

## Publications Arising From This Research

Mann, V., Large, A.T., Khan, S., Malik, Z. and Connock, M.J. (1989) Aromatic alcohol oxidase : a new membrane-bound  $H_2O_2$  generating enzyme in the alimentary tissues of the slug *Arion ater*. J. Exp. Zool. **251** : 265-274.

Large, A.T. and Connock, M.J. (1993) Aromatic alcohol oxidase and aldehyde oxidase activities in the digestive gland of three species of terrestrial gastropod. Comp. Biochem. Physiol. **104B** : 489-491.

Large, A.T., Jones, C.J.P. and Connock, M.J. (1993) The association of mannitol oxidase with a distinct organelle in the digestive gland of the terrestrial slug *Arion ater*. Protoplasma **175** : 93-101.

Large, A.T. and Connock, M.J. (1994) Centrifugal evidence for the association of mannitol oxidase with distinct organelles ("mannosomes") in the digestive gland of several species of terrestrial gastropod mollusc. Comp. Biochem. Physiol. **107A** : 621-629.

Large, A.T. and Connock, M.J. (1994) Hydrogen peroxide generating alcohol and aldehyde oxidases in the giant african land snail, *Achatina fulica*. J. Exp. Zool. **270** : 445-450.

# Acknowledgments

I would to thank Dr. Martin Connock for supervising this project. I am also grateful for his genuine enthusiasm for the subject.

I would like to thank Dr. Carolyn J.P. Jones (University of Manchester) for the excellent electron micrographs presented in this thesis.

I would also like to thank Dr. Chris Perry (University of Wolverhampton) for the synthesis of 2,4,6-tribromo-3-hydroxy-benzoic acid.

I would also like to thank the following for their help and advice over the past six years :- (in alphabetical order)

Dr. Daron Fincham, Richard Homfray, Malcolm Inman, Meena Jefferies, Sultan Khan, Zulfiqar Malik, Vincent Mann, Dr. Chara Nallaiah, Mark Powell, Dr. Raul Sutton.

# Abbreviations

Wherever possible the use of abbreviations has been avoided or the abbreviation has been explained in the text. However the following may be encountered :-

AA	4-Aminoantipyrine (4-Aminophenazone)
l-DCF	<i>leuco</i> -2,7-Dichlorofluoroscini
EDTA	Ethylenediaminetetra-acetic acid
FAD	Flavin adenine dinucleotide
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP <sup>+</sup>	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PAGE	Polyacrylamide gel electrophoresis
PMS	Phenazine methosulphate
PNS	Post nuclear supernatant
PQQ	Pyrroloquinoline quinone
r.p.m.	Revolutions per minute
SDS	Sodium dodecyl sulphate
TBHBA	2,4,6-Tribromo-3-hydroxy-benzoic acid



# Aromatic Alcohol Oxidase, Aldehyde Oxidase and Mannitol Oxidase in Terrestrial Gastropods.

## Abstract

Several unusual hydrogen peroxide generating oxidase activities have been investigated in the alimentary tissues of terrestrial gastropod molluscs.

A membrane-bound aromatic alcohol oxidase activity has been demonstrated in the digestive gland (hepatopancreas) of four families of gastropod (*Arionidae*, *Limacidae*, *Helicidae* and *Achatinidae*). Aromatic alcohol oxidase activity has only previously been reported in basidiomycete fungi where the activity is soluble, extracellular and probably involved in lignin biodegradation. The gastropod activity has been solubilised in non-ionic detergents and the solubilised activity tested with a variety of potential substrates. In three species (*Arion ater*, *Limax flavus* and *Helix aspersa*) cinnamyl alcohol (1mM, *cis/trans* mixed isomers) was found to be the most favoured substrate. In the fourth, *Achatina fulica*, the unsaturated aliphatic alcohol crotyl alcohol (2-buten-1-ol, *cis/trans* mixed isomers) was found to be the most favoured substrate at 1mM although cinnamyl alcohol was also readily oxidised. All four species could oxidise crotyl alcohol although only the activity from *A. fulica* could oxidise saturated aliphatic primary alcohols. Alcohols up to C<sub>16</sub> (*n*-hexadecyl) were utilised by this activity. Subcellular fractionation experiments showed aromatic alcohol oxidase activity to be localised in the endoplasmic reticulum.

Aldehyde oxidase activity was considered in relation to its possible role in the further utilisation of the products of aromatic alcohol oxidase. It is a soluble cytosolic activity. Gastropod aldehyde oxidase is active with a variety of aldehydes but not with purines unlike the mammalian enzyme. Salicylaldehyde was found to be a specific inhibitor of the *A. ater* and *L. flavus* activities.

D-Mannitol oxidase thus far has only been reported in gastropod molluscs. Activity from the four species mentioned above is very similar with regard to substrate preference and apparent K<sub>m</sub> values. D-Mannitol, D-arabitol and to a lesser extent sorbitol (D-glucitol) are oxidised. Subcellular fractionation of *Arion ater* digestive gland showed mannitol oxidase to be localised in a low density membrane distinct from mitochondria, peroxisomes and endoplasmic reticulum. When enriched by successive rate dependent and isopycnic sucrose gradient centrifugation the mannitol oxidase fractions were analysed by electron microscopy. This revealed the predominance of tubular structures typically of seven tubules (each of approx. 50nm in diameter) in a hexagonal 6 + 1 arrangement surrounded by a smooth membrane. An interim term "mannosome" has been proposed for these structures. S.D.S.-P.A.G.E. analysis revealed a single predominant polypeptide of 68-69kDa (the published value for partially purified mannitol oxidase) in the mannitol oxidase-enriched fractions. Analytical sucrose gradient centrifugation techniques provided evidence for the presence of "mannosomes" in *Helix aspersa* although the situation is less clear for *Achatina fulica* and *Limax flavus*.

# 1. INTRODUCTION

## 1.1. General Introduction

This thesis describes the subcellular localisation and initial characterisation of three unusual hydrogen peroxide-generating oxidase activities found in terrestrial gastropod molluscs. The three activities described are aromatic alcohol oxidase, aldehyde oxidase and mannitol oxidase. These activities were observed and studied in representative species of four families of gastropod. The temperate slug species *Arion ater*, *Arion lusitanicus* (family : *Arionidae*) and *Limax flavus* (family : *Limacidae*) were used in this study. The common garden snail *Helix aspersa* (family : *Helicidae*) and the tropical giant african land snail *Achatina fulica* (family : *Achatinidae*) were also studied.

### 1.1.1. History of the Study

D-Mannitol oxidase was first reported by Vorhaben *et al* (1980) in digestive gland mitochondrial preparations from *Helix aspersa*. Subsequently the activity was found to be localised mainly in digestive tissues (Vorhaben *et al* 1984). The membrane-bound enzyme was later solubilised and partially purified (Vorhaben *et al* 1986). A later study (Malik *et al* 1987) demonstrated mannitol oxidase in the tissues of the terrestrial slug *Arion ater*. Subcellular fractionation showed that mannitol oxidase was localised in a low-density membrane rather than mitochondria.

The histochemical localisation of mannitol oxidase was being investigated in this laboratory using glutaraldehyde-fixed *Arion ater* tissue sections when a very active hydrogen peroxide-generating activity was observed in the control sections (i.e.: those lacking the substrate, mannitol). This activity was presumably utilising the exogenously supplied glutaraldehyde used to fix the sections and was found to be cytosolic aldehyde oxidase. This observation prompted the search for other hydrogen peroxide-generating oxidases in the



tissues of this slug. This search led to the observation of alcohol-dependent hydrogen peroxide production in tissue homogenates. It was later found that the hydrogen peroxide was being produced by a particulate aromatic alcohol oxidase and that the activity was as much as two orders of magnitude greater with some aromatic alcohols when compared to the activity with ethanol or propan-1-ol.

Thus far mannitol oxidase appears to be unique to molluscs. Particulate aromatic alcohol oxidase may also prove to be unique although soluble oxidases with similar activity are well characterised in lignolytic fungi. Cytosolic aldehyde oxidase has been fully characterised in mammals but this enzyme is functionally a heterocycle oxidase; molluscan aldehyde oxidase is considered here in relation to its possible role in the oxidation of the products of aromatic alcohol oxidase.

### 1.1.2. Aims and Scope of Investigation

Mannitol oxidase and aromatic alcohol oxidase are examples of particulate hydrogen peroxide-generating activities. Oxidases are often peroxisomal; notable examples include yeast alcohol oxidases and urate oxidase in non-primate mammals. Oxidases and other hydrogen peroxide-generating proteins are however localised at many subcellular sites (this topic is considered in greater detail in section 1.2.). Therefore one aim of this study has been to investigate the subcellular localisation of mannitol oxidase and aromatic alcohol oxidase in *Arion ater* digestive gland. Various techniques have been used to positively identify the low-density membranes that bear mannitol oxidase. The question of the utilisation or detoxification of hydrogen peroxide at the subcellular locales of these oxidases arises but this is beyond the experimental remit of this study. Subcellular fractionation results from several other species of gastropod have been compared to *Arion ater*.

Particulate aromatic alcohol oxidase is clearly very different to the soluble fungal enzyme. Part of this study has been to characterise this



activity (particularly with respect to the substrate specificity) using a fairly crude preparation in several species of gastropod and also to begin to purify the protein. A sixty-fold enrichment of the activity has been achieved relative to the original homogenate but this still represents a heterogeneous mixture of proteins. Soluble cytosolic aldehyde oxidase is a well characterised activity in mammals. The molluscan enzyme has been considered here in several species with regard to its substrate specificity especially those substrates that are the likely products of aromatic alcohol oxidase. The soluble fraction of digestive gland homogenate has been used as a source of aldehyde oxidase activity and no further purification attempted. Mannitol oxidase has not been purified by conventional protein purification techniques in this study. The purification of the mannitol oxidase-bearing organelle has instead been a primary aim. Subcellular fractions have been used as a crude source of mannitol oxidase activity and this has been used to compare the substrate specificities of this activity in several species.

These three activities are in many ways unlike other enzymes with a similar function. The various alcohol, aldehyde and sugar alcohol oxidising enzymes found in nature have been considered in sections 1.3. (alcohol-oxidising), 1.4. (aldehyde-oxidising) and 1.5. (sugar alcohol-oxidising).

The information gained from the characterisation of these three activities will hopefully provide information on the metabolism of the dietary components that these enzymes utilise. The ultimate fate of alcohols, aldehydes and sugar alcohols ingested by these animals has not been fully investigated. The exact role of these activities *in vivo* and how they interact with the rest of the gastropod metabolism remains unknown. The functions of the mannitol oxidase-bearing organelle also awaits full elucidation.

## 1.2. The Generation and Detoxification of Hydrogen Peroxide and Other Reactive Oxygen Species

Oxygen ( $O_2$ ) can be reduced to the reactive superoxide anion ( $O_2^{\cdot-}$ ) by a single-electron transfer, to hydrogen peroxide ( $H_2O_2$ ) by a two-electron transfer or fully reduced to water ( $H_2O$ ). Although hydrogen peroxide is not as reactive as the superoxide anion it can give rise to a very reactive oxygen species, the hydroxyl radical ( $\cdot OH$ ), in the presence of redox-active transition metals (for example  $Fe^{2+}$ ) (Pahl and Baeurele 1994). Superoxide can also give rise to a number of more reactive oxygen species including the hydroxyl radical ( $\cdot OH$ ), the perhydroxyl radical ( $\cdot OH_2$ ) and singlet oxygen ( $^1O_2$ ) (Yu 1994). Hydrogen peroxide and superoxide are generated in biological systems by oxidases, flavoproteins and other redox-proteins such as those found in the mitochondrial respiratory chain. These reactive oxygen intermediates are generated in the cytosol and by several subcellular membranes.

Reactive oxygen species can cause cellular damage so the levels of these physiological products must be controlled. Superoxide dismutase catalyses the conversion of superoxide into hydrogen peroxide and molecular oxygen. Hydrogen peroxide is broken down into water and molecular oxygen by catalase. These two detoxifying activities are found in virtually all aerobic organisms. Glutathione peroxidase and antioxidant vitamins (for example C and E) also protect the cell against potential damage by reactive oxygen intermediates (Yu 1994).

### 1.2.1. Subcellular Sites of Hydrogen Peroxide Generation

#### 1.2.1.1. Mitochondria

In mitochondria hydrogen peroxide is generated by components of the respiratory chain, flavoprotein dehydrogenases and by specific



oxidases. The generation of hydrogen peroxide by the respiratory chain depends on the metabolic state of the mitochondria. Under physiological conditions hydrogen peroxide generation is greatest where the components of the respiratory chain are highly reduced such as in the presence of NAD<sup>+</sup>-linked substrates and succinate. Under these conditions hydrogen peroxide generation accounts for only about 2% of the total oxygen consumption of the mitochondria. In isolated mitochondria hydrogen peroxide generation is increased several-fold by antimycin A, a respiratory inhibitor. The superoxide anion rather than hydrogen peroxide is generated (at the cytochrome b-ubiquinone stage of the chain) but this is rapidly converted to hydrogen peroxide by mitochondrial manganese-dependent superoxide dismutase (Chance *et al* 1979, Yu 1994). It has been reported (Boveris *et al* 1976) that ubiquinone is essential for the generation of superoxide (and therefore hydrogen peroxide) by the respiratory chain. Sub-mitochondrial particles that are devoid of auxiliary flavoproteins, inhibited by antimycin A and supplied with succinate or NAD<sup>+</sup>-linked substrate require ubiquinone to produce superoxide. It was proposed that the reduction products of ubiquinone (ubiquinol and ubisemiquinone) react non-enzymatically with molecular oxygen to produce superoxide.

Mammalian dihydro-orotate dehydrogenase is a mitochondrial flavoprotein involved in the biosynthesis of uridine monophosphate. It has a similar mode of entry into the respiratory chain as succinate dehydrogenase (also a flavoprotein). Foreman and Kennedy (1975) reported that superoxide was produced by the dehydrogenase *in situ* without feeding into the respiratory chain; succinate dehydrogenase does not produce superoxide itself but succinate-dependent superoxide generation is contingent upon entry of the electrons derived from succinate oxidation to the respiratory chain. It has more recently been shown dihydro-orotate dehydrogenase (purified to homogeneity) has hydrogen peroxide-generating oxidase activity *in vitro* (Lakaschus *et al* 1991). Hydrogen peroxide is also produced by mitochondrial oxidases such as monoamine oxidase (Baudhuin *et al* 1964). Some of the hydrogen peroxide generated by the mitochondria is probably able to diffuse into the surrounding cytosol (Yu 1994).

### 1.2.1.2. Peroxisomes

Many hydrogen peroxide generating oxidative enzymes are compartmentalised with catalase in organelles called peroxisomes. L-Hydroxy-acid oxidase is localised in peroxisomes in vertebrates and plants. Urate oxidase is another peroxisomal enzyme present in many species (but not primates) (Masters and Holmes 1977). D-Amino acid oxidase is a peroxisomal enzyme found in many animals including gastropod molluscs (Malik *et al* 1987). The protein content of the peroxisomes of yeasts grown on methanol is composed almost entirely of just two proteins, alcohol oxidase and catalase (see section 1.3.5.). Superoxide may be generated in peroxisomes by xanthine oxidase although the a peroxisomal localisation for this oxidase is not certain; some peroxisomes do however contain copper/zinc-dependent superoxide dismutase (Keller *et al* 1991).

Although peroxisomes contain very high levels of catalase activity some of the hydrogen peroxide generated might escape into the surrounding media. Although the theoretical leakage is only about 2% of the total hydrogen peroxide generated in practice isolated peroxisomes leak up to 40%. However this is probably due mainly to damage incurred during isolation (Chance *et al* 1979).

### 1.2.1.3. Endoplasmic Reticulum

The membranes of the endoplasmic reticulum generate hydrogen peroxide via flavoproteins (for example NADPH-cytochrome c reductase), specific oxidases (for example diamine oxidase) and cytochromes (for example cytochrome P450) (Chance *et al* 1979). This thesis describes an aromatic alcohol oxidase that is most likely localised in the endoplasmic reticulum in gastropod molluscs. An active NADPH-cytochrome c reductase was also found associated with these membranes therefore the endoplasmic reticulum has a significant potential for hydrogen peroxide generation in these animals.



In general the endoplasmic reticulum can also generate measurable quantities of superoxide. The NADPH-cytochrome P450 reductase system probably is responsible for much of this superoxide as the auto-oxidation of cytochrome P450 is known to cause the formation of superoxide (Ramasarma 1982).

#### 1.2.1.4. Cytosol

Cytosolic oxidases such as aldehyde oxidase generate hydrogen peroxide. Xanthine oxidase also generates some superoxide although cytosolic copper/zinc-dependent superoxide dismutase would rapidly convert this to hydrogen peroxide. The cytosolic hydrogen peroxide levels would also include that generated at various subcellular sites and released into the cytosol (Chance *et al* 1979).

#### 1.2.1.5. Other Organelles

Nuclei also generate hydrogen peroxide via the cytochromes located in the nuclear membrane. Plasma membranes in mammalian liver cells contain a NADH-oxidoreductase with a potential for hydrogen peroxide generation that is distinct from other similar microsomal enzymes (Ramasarma 1982).

This study has shown that hydrogen peroxide is generated at several subcellular sites in the digestive gland of gastropod molluscs namely cytosol (aldehyde oxidase), endoplasmic reticulum (aromatic alcohol oxidase), low-density membrane (mannitol oxidase) and possibly plasma membrane (semicarbazide-sensitive amine oxidase). Peroxisomal D-amino acid oxidase has also been demonstrated in these animals (Malik *et al* 1987). It is probable that the mitochondrial respiratory chain and nuclear membranes are not dissimilar to most other organisms and also generate hydrogen peroxide. Therefore the potential for hydrogen peroxide production is present at many subcellular sites in these animals.

## 1.2.2. The Detoxification of Hydrogen Peroxide and Related Oxygen Species

### 1.2.2.1. Toxicity of Reactive Oxygen Species

Hydrogen peroxide and superoxide are physiological products and low levels are naturally present in the cell. Hydrogen peroxide can be a useful metabolite as well as a potentially harmful one. It is produced by mammalian leucocytes during phagocytosis possibly to help kill bacteria (Chance *et al* 1979). It is also important in alcohol oxidation by yeast and possibly liver cells where it is utilised by catalase to peroxidatically oxidise ethanol (see section 1.3.1.). However both hydrogen peroxide and superoxide give rise to several extremely harmful radicals such as the hydroxyl and perhydroxyl radicals. The levels of reactive oxygen species must therefore be controlled otherwise various types of cellular damage could occur (Yu 1994).

Free radicals generated from hydrogen peroxide and superoxide can react with various cellular components causing protein denaturation and lipid peroxidation. The toxic effects of lipid peroxidation include membrane instability, alteration to their permeability and enzyme inhibition (Chance *et al* 1979). Damage can also be caused to D.N.A. particularly mitochondrial D.N.A. which is both exposed to a higher concentration of reactive oxygen species and has a less effective repair system (Yu 1994).

### 1.2.2.2. Antioxidant Enzymes

Superoxide dismutase, catalase and glutathione peroxidase are responsible for the enzymatic detoxification of superoxide and hydrogen peroxide in the cell. Superoxide dismutase is localised in mitochondria (manganese-dependent), the cytosol (copper/zinc-dependent) and peroxisomes (copper/zinc-dependent). It converts superoxide to hydrogen peroxide and molecular oxygen. Catalase is mostly peroxisomal but may also be cytosolic and catalyses the breakdown of hydrogen peroxide to water and molecular oxygen.

### Superoxide Dismutase



### Catalase



### Glutathione Peroxidase



### Glutathione Reductase



**Figure 1.2.2.2. A summary of the reactions of various antioxidant enzymes**

GSH = reduced glutathione

GSSG = oxidised glutathione

ROOH = alkyl peroxide



Selenium-dependent glutathione peroxidase is a cytosolic activity that couples the oxidation of glutathione to the reduction of hydrogen peroxide and other hydroperoxides (Yu 1994). The cytosol is well buffered with reduced glutathione making it a strongly reducing environment in the presence of glutathione peroxidase. Oxidised glutathione is recycled by NADPH-dependent glutathione reductase to maintain the level of reduced glutathione (Chance *et al* 1979). The reactions of these enzymes are summarised in figure 1.2.2.2.

A study by Livingston *et al* (1992) investigated the subcellular localisation of antioxidant enzyme activities in the digestive gland of the bivalve mollusc *Mytilus edulis*. Superoxide dismutase activity was found to be mainly cytosolic (copper/zinc dependent) with some activity also localised in the mitochondria (due to manganese-dependent superoxide dismutase). Catalase was considered to be mainly peroxisomal; the activity in the cytosolic fraction was accounted for by the probable damage caused to the peroxisomes during fractionation. Selenium-dependent glutathione peroxidase was found to be mainly cytosolic with some activity associated with the endoplasmic reticulum. These antioxidant enzyme activities have complementary function and subcellular localisations; catalase removes hydrogen peroxide produced by oxidases in the peroxisomes and glutathione peroxidase removes hydrogen peroxide and hydroperoxides in the cytosol. Distinct forms of superoxide dismutase are responsible for detoxifying superoxide in the cytosol and the mitochondria.

The regulation of the gene expression of antioxidant enzymes in prokaryotes (for example *Escherichia coli*) has recently been elucidated (Pahl and Baeuerle 1994). The oxyR regulon encodes for enzymes such as catalase and alkyl hydroperoxide reductase. It is regulated by a transcription factor (OxyR) which is activated by hydrogen peroxide. The expression of genes encoding for superoxide dismutase is increased by a response cascade. SoxR is a superoxide sensor that when activated by superoxide induces the transcription of the SoxS gene. This in turn increases the expression of defensive proteins such as manganese-dependent superoxide dismutase. A known transcription factor (Human NF- $\kappa$ B) in higher eukaryotes responds to



reactive oxygen species and activates the expression of genes that encode for defensive agents against pathological conditions.

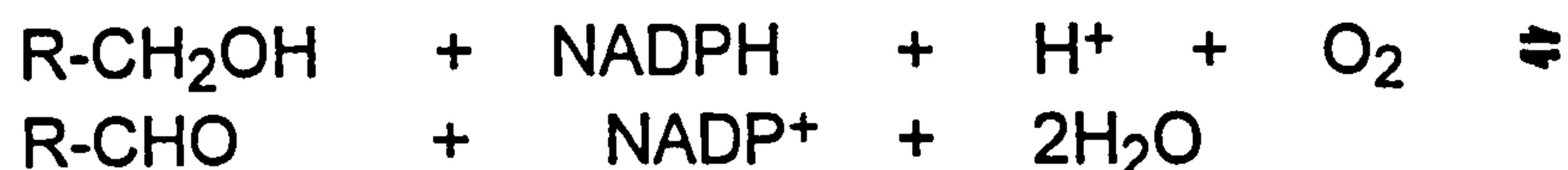
#### 1.2.2.3. Other Cellular Defences Against Reactive Oxygen Species

In addition to the antioxidant enzymes described previously cells are also protected against the deleterious effects of reactive oxygen species by antioxidant compounds and secondary enzyme defences. Many compounds scavenge hydroxyl radicals including glucose, uric acid, mannitol and benzoic acid. Water-soluble vitamin C and membrane-soluble vitamin E both scavenge many of the oxygen radicals (superoxide, hydroxyl radical etc.) as well as other free radicals. Proteolytic and lipolytic enzymes act as a secondary enzymatic defence by breaking down oxidatively-damaged proteins and peroxidised phospholipid (Yu 1994).

## 1 Catalase



## 2 Microsomal Ethanol Oxidising System (MEOS)



## 3 NAD(P)<sup>+</sup>-dependent Dehydrogenases



## 4 NAD(P)<sup>+</sup>-independent (PMS-linked) Dehydrogenases



## 5 Oxidases



**Figure 1.3. A summary of the reactions of various alcohol oxidising enzymes**

Cyt = cytochrome  
(ox) = oxidised form  
(red) = reduced form

## 1.3. Alcohol Oxidising Enzymes

Monohydric primary alcohols are widespread physiological substrates. For example ethanol is a fermentation product of yeast and may be found naturally in partially decomposed plant matter. Methanol can be utilised as the sole carbon source by many micro-organisms. Methanol is the initial metabolite in methane utilisation and is produced during the enzymatic demethoxylation of aromatic compounds. Aromatic alcohols are the building blocks of lignin in plants, one of the most abundant naturally occurring polymers. Aromatic alcohols such as sinapyl, coumaryl and coniferyl alcohols are stored in plants as the 4-*O*- $\beta$ -D-glucopyranosides prior to lignification in plant tissues (Savidge and Udagama-Randeniya 1992). Benzyl alcohol occurs as an intermediate in the biodegradation of aromatic compounds by bacteria. Long-chain aliphatic alcohols (fatty alcohols) are found in waxes produced by plants and are also the initial metabolites in alkane degradation by bacteria and yeasts.

Alcohol oxidation is therefore important to a wide range of organisms. There are many enzymes and enzyme systems responsible for the oxidation of alcohols to their corresponding aldehydes. They can be broadly categorised into five main groups (Cardemil 1978) :- 1) catalase acting peroxidatically, 2) microsomal ethanol oxidising system (MEOS) of mammalian liver, 3) NAD(P)<sup>+</sup>-linked dehydrogenases, 4) NAD(P)<sup>+</sup>-independent (phenazine methosulphate (PMS) linked) dehydrogenases and 5) alcohol oxidases. The reactions of each type are summarised in figure 1.3. and each category will be considered in turn.

### 1.3.1. Catalase

Catalase is a virtually ubiquitous enzyme that is absent only from strict anaerobes and a very few aerobic bacteria. It is primarily responsible for the enzymatic decomposition of hydrogen peroxide to water and molecular oxygen (Deisseroth and Dounce 1970). As well as hydrogen peroxide it may also bind several small molecules including methanol, ethanol and formaldehyde which are oxidised in a peroxidatic reaction. Methanol and ethanol are readily oxidised but propan-1-ol and butan-1-ol are only slowly oxidised (Chance 1947). Although possibly of only limited significance in mammals this activity is important in certain yeast peroxisomes where catalase utilises the hydrogen peroxide produced by alcohol oxidase (also localised in the peroxisomes) to oxidise methanol (Roggenkamp *et al* 1975)(see section 1.3.5.).

### 1.3.2. Microsomal Ethanol Oxidising System

The ability of mammalian hepatic microsomes to oxidise methanol, ethanol, propan-1-ol and butan-1-ol has aroused interest because of its possible role in human ethanol metabolism (Lieber and DeCarle 1968). Microsomal ethanol oxidation has been shown to be completely independent of both cytosolic NAD<sup>+</sup>-dependent alcohol dehydrogenases and catalase. The system requires NADPH / H<sup>+</sup> and molecular oxygen to promote alcohol oxidation (Teschke *et al* 1975). A distinct isoenzyme of cytochrome P450 (isoenzyme 3a) has been shown to be a component of the microsomal ethanol oxidising system (M.E.O.S.) in rabbit liver microsomes (Koop *et al* 1982). Ethanol oxidation has been extensively studied in a mutant strain of deermice that lacks NAD<sup>+</sup>-dependent alcohol dehydrogenase activity (ADH<sup>-</sup>). These animals can however metabolise ethanol at up to 60% the rate of deermice with alcohol dehydrogenase (ADH<sup>+</sup>). It is strongly debated whether the M.E.O.S. or catalase/H<sub>2</sub>O<sub>2</sub> is more significant in these ADH<sup>-</sup> animals (Tagaki *et al* 1986, Handler *et al* 1986).



Long-(polypeptide)-chain Alcohol Dehydrogenase Family  
(Jörnvall *et al* 1987)

Alcohol dehydrogenase (dimeric) - mammalian liver  
Alcohol dehydrogenase (dimeric) - plants  
Alcohol dehydrogenase (tetrameric) - *Saccharomyces cerevisiae*  
Sorbitol dehydrogenase (tetrameric) - mammalian liver

Short-(polypeptide)-chain Alcohol Dehydrogenase Family  
(Persson *et al* 1991)

Alcohol dehydrogenase - *Drosophila melanogaster*  
Ribitol dehydrogenase - *Klebsiella aerogenes*  
Sorbitol-6-phosphate dehydrogenase - *Escherichia coli*  
Glucose dehydrogenase - *Bacillus megaterium*  
17- $\beta$ -Hydroxysteroid dehydrogenase - Human

Iron-activated Alcohol Dehydrogenases  
(Williamson and Paquin 1987)

Alcohol dehydrogenase - *Zymomonas mobilis*  
Alcohol dehydrogenase - *Saccharomyces cerevisiae*

Table 1.3.3. - A summary of the families of ethanol dehydrogenases (ethanol-NAD<sup>+</sup>-oxidoreductases) and related enzymes

### 1.3.3. NAD(P)<sup>+</sup>-linked Dehydrogenases

This is a very large and varied group of enzymes. In addition to ethanol oxidising enzymes there are enzymes specific for fatty alcohols, aromatic alcohols (benzyl alcohol etc.) and lignin monomeric alcohols (coniferyl alcohol etc.).

Soluble ethanol dehydrogenases (ethanol-NAD<sup>+</sup> oxidoreductases) can be grouped into three families :- zinc-containing long-(polypeptide)-chain alcohol dehydrogenases of animals, plants and yeasts, short-(polypeptide)-chain alcohol dehydrogenases found in insects (and related enzymes in other organisms) and iron-activated alcohol dehydrogenases of some micro-organisms (see table 1.3.3.).

The long-(polypeptide)-chain alcohol dehydrogenase family of enzymes contain zinc and include mammalian, plant, fungal and yeast ethanol dehydrogenases as well as mammalian sorbitol dehydrogenase. Although there is considerable variation between enzymes from the various types of organisms they are clearly related and have sub-units of approximately 42kDa (350 - 375 residues). The active enzymes are either dimeric (mammalian and plant alcohol dehydrogenase) or tetrameric (yeast alcohol dehydrogenase and mammalian sorbitol dehydrogenase) (Jörnvall *et al* 1987). There is variation between the substrate specificities of enzymes from various species but ethanol is always a good substrate. Benzyl alcohol is a moderate substrate for mammalian alcohol dehydrogenase but yeast and plant enzymes are inactive with this substrate (Tsai *et al* 1987).

The family of short-(polypeptide)-chain alcohol dehydrogenases includes *Drosophila* alcohol dehydrogenase as well as functionally different enzymes in animals and bacteria (see table 1.3.3.). This family of enzymes differs from the long-chain alcohol dehydrogenases in lacking a metal cofactor and having a smaller subunit size of approximately 29kDa (250 residues). Although *Drosophila* alcohol dehydrogenase is active with ethanol secondary alcohols are also

good substrates (Ribas de Pouplane *et al* 1991, Persson *et al* 1991). The final family of alcohol dehydrogenases is much smaller. The fermentative bacteria *Zymomonas mobilis* has an iron-activated alcohol dehydrogenase that is distinct from the enzyme families described above (Scopes 1983). A homologous alcohol dehydrogenase has been found in the yeast *Saccharomyces cerevisiae* (Williamson and Paquin 1987).

As well as the ethanol-oxidising enzymes above there are NAD<sup>+</sup>-linked dehydrogenases specific for other primary alcohol substrates. Benzyl alcohol dehydrogenase is a plasmid-encoded enzyme of the bacteria *Pseudomonas putida* and is part of the pathway for the oxidative degradation of toluene and xylene. Although it lacks zinc or other metal cofactor it is related to the long-(polypeptide)-chain family of alcohol dehydrogenases and is a dimeric protein with subunits of 42kDa (Shaw *et al* 1993). A similar enzyme activity (not plasmid encoded) has been reported in *Acinetobacter calcoaceticus* (a *P. putida* strain) but the protein is tetrameric with subunits of 39.7kDa (MacKintosh and Fewson 1988). The lignolytic bacteria *Rhodomonas erythropolis* and *Xanthomonas* sp. have a NAD<sup>+</sup>-dependent dehydrogenase specific for aromatic alcohols bearing the  $\alpha$ - $\beta$ -unsaturated side-chain of lignin monomers (coniferyl, sinapyl and coumaryl alcohols) and dilignols (dehydroconiferyl alcohol and guaiacyl-glycerol- $\beta$ -coniferyl ether) (Jaeger *et al* 1981, Kern *et al* 1984).

Alkane-grown yeasts incorporate alkanes by first hydroxylating alkanes to the corresponding alcohols via a cytochrome P450 and a NADPH-cytochrome P450 reductase system. A particulate NAD<sup>+</sup>-dependent alcohol dehydrogenase is present in alkane-utilising yeasts (for example *Candida tropicalis*, *Candida lipolytica* and alkane-adapted *Saccharomyces cerevisiae*) specific for medium- and long-chain (fatty) alcohols; aldehyde dehydrogenase subsequently oxidises the fatty aldehyde to the corresponding fatty acid which is either degraded by peroxisomal  $\beta$ -oxidation or used in lipid biosynthesis in microsomes and mitochondria (Ueda and Tanaka 1990). This activity can be clearly distinguished from ethanol oxidising dehydrogenases from the organism. In *Candida tropicalis* activity peaks with decan-1-ol and in



ENZYME	SPECIES	M.W.	SUBUNIT COMPOSITION	CO-FACTOR	REFERENCE
Alcohol dehydrogenase	<i>Gluconobacter suboxydans</i>	150 kDa	85 kDa ( dehydrogenase ) 49 kDa (cytochrome ) 14.4 kDa	PQQ	Ameyama and Adachi ( 1982a )
Alcohol dehydrogenase	<i>Acetobacter aceti</i>	149 kDa	63 kDa ( dehydrogenase ) 44 kDa ( cytochrome) 29 kDa 13.3 kDa	PQQ	Ameyama and Adachi ( 1982a )
Aldehyde dehydrogenase	<i>Gluconobacter suboxydans</i>	140 kDa	86 kDa ( dehydrogenase ) 55 kDa ( cytochrome )	PQQ	Ameyama and Adachi ( 1982b )
Aldehyde dehydrogenase	<i>Acetobacter aceti</i>	137 kDa	78 kDa ( dehydrogenase) 45 kDa ( cytochrome ) 14kDa	PQQ	Ameyama and Adachi ( 1982b )
Sorbitol dehydrogenase	<i>Gluconobacter suboxydans</i>	131 kDa	63 kDa ( dehydrogenase ) 51 kDa ( cytochrome ) 17kDa	Covalently-bound flavin ( in dehydrogenase subunit ) and unidentified quinone	Shinagawa <i>et al</i> (1982)

Table 1.3.4. - NAD(P)<sup>+</sup>-independent (cytochrome-linked) dehydrogenases of acetic acid bacteria

*Saccharomyces cerevisiae* with octan-1-ol (Lebault *et al* 1969, Roche and Azoulay 1969). A similar activity is found in the bacteria *Pseudomonas aeruginosa* grown on alkanes; this activity peaks with heptan-1-ol (Azoulay and Heydeman 1963).

#### 1.3.4. NAD(P)<sup>+</sup>-independent Alcohol Dehydrogenases

In methylotrophic, alkane-degrading, alcohol-grown and acetic acid bacteria there are a class of alcohol dehydrogenases that are NAD(P)<sup>+</sup>-independent. These enzymes have an unusual cofactor, pyrroloquinoline quinone (PQQ) and will utilise a variety of electron acceptors including phenazine methosulphate (PMS); sometimes this group of activities is referred to as PMS-dependent dehydrogenases. The electron-acceptor *in vivo* is most probably a cytochrome. In methylotrophs the electron-acceptor has been identified as cytochrome c<sub>L</sub> (Day and Anthony 1990).

The NAD(P)<sup>+</sup>-independent methanol dehydrogenases of methylotrophs (*Hyphomicrobium* X and *Methylobacterium extoquens* AM1) are dimeric proteins (60kDa subunits) and are activated by ammonium salts and primary amines. Alcohol dehydrogenases of alcohol-grown pseudomonads are of two types :- PQQ-containing monomeric enzymes of 100kDa (*P. aeruginosa*) which are ammonium activated and quinohaem proteins of 70kDa which contain PQQ and haem C (*P. testosteroni*). This latter type is distinguished by not being activated by ammonium ions. Both types are much less active with methanol than ethanol (Groen and Duine 1990).

The acetic acid bacteria *Acetobacter aceti* and *Gluconobacter suboxydans* have a PQQ-containing ethanol dehydrogenase located on the outer surface of the cytoplasmic membrane. This is linked via a cytochrome to the respiratory chain. This is a common feature of several NAD(P)<sup>+</sup>-independent dehydrogenases from acetic acid bacteria (see table 1.3.4.) (Inoue *et al* 1989, Ameyama and Adachi 1982a).

	SPECIES	NATIVE M.W.	SUBUNIT M.W.	pH OPTIMUM	PROSTHETIC GROUP	REFERENCE
YEASTS	<i>Hansenula polymorpha</i>	669 kDa (homo-octamer)	83 kDa	8.0 -9.0	FAD	Kato <i>et al</i> 1976
	<i>Kloeckera sp.</i>	673 kDa (homo-octamer)	83 kDa	8.0 -9.0	FAD	Kato <i>et al</i> 1976
	<i>Candida boidinii</i>	600 kDa (homo-octamer)	74 kDa	7.5 - 9.5	FAD	Sahm and Wagner 1973
FUNGI	<i>Polyporus sp.</i>	> 300 kDa -	85 kDa	7.0 - 9.0	FAD	Janssen and Ruelius 1968
	<i>Poria contigua</i>	610 kDa (homo-octamer)	79 kDa	-	FAD	Bringer <i>et al</i> 1979
PLANT	<i>Tanacetum vulgare</i>	180 kDa (hetero-dimer)	94 kDa 75 kDa	5.5 - 9.0	Flavin	Banthorpe <i>et al</i> 1976

Table 1.3.5.a) Properties of various aliphatic alcohol oxidases



### 1.3.5. Alcohol Oxidases

Hydrogen peroxide generating alcohol oxidases (alcohol : oxygen oxidoreductases) are found in yeasts, fungi, higher plants and, as described in this thesis, gastropod molluscs. Methanol oxidases are the most studied group and are found in yeasts and basidiomycete fungi. There are also alcohol oxidases specific for aromatic, long-chain and unsaturated aliphatic alcohols.

Alcohol (methanol) oxidase is a key enzyme in methanol utilisation by methylotrophic yeasts and produces formaldehyde and hydrogen peroxide. It has been reported in many species including *Hansenula polymorpha*, *Pichia pastoris*, *Candida boidinii* and *Kloeckera* sp. (van der Klei et al 1990). In general yeast alcohol oxidases are homooctameric proteins with a FAD prosthetic group; some properties of these enzymes are summarised in table 1.3.5.a). Methanol is always a good substrate for these enzymes but activity decreases rapidly with chain-length (table 1.3.5.b)). In *Candida boidinii* alcohol oxidase and catalase are localised in peroxisomes as crystalloid inclusions and these two enzymes account for most of the protein of the organelle. Catalase is therefore well placed to utilise the hydrogen peroxide produced by alcohol oxidase to peroxidatically oxidise alcohol (see section 1.3.1.) (Roggenkamp et al 1975).

Similar alcohol (methanol) oxidases are found in a number of white-rot and brown-rot basidiomycete fungi including *Poria contigua*, *Polyporus* sp. B191039, *Polyporus obtusus*, *Polyporus versicolor*, *Lenzites trabea*, *Radulum casaerium* and *Irpex flavus* (Janssen and Ruelius 1968, Kerwin and Ruelius 1969, Bringer et al 1979). These are also flavoproteins of similar subunit size to the yeast enzymes and also oxidise lower primary alcohols (see tables 1.3.5.a) and 1.3.5.b)). However these enzymes are immunologically different from yeast enzymes. Enzymes from brown-rot fungi (*Poria contigua*) and white-rot fungi (*Polyporus* sp.) are also immunologically different from each other. Their function *in vivo* appears to be the oxidation of methanol produced by the enzymatic demethoxylation of aromatic compounds,

SUBSTRATE	SPECIES						
	YEASTS		FUNGI			PLANT	
	<i>Candida boidinii</i>	<i>Hansenula polymorpha</i>	<i>Kloeckera</i> sp.	<i>Kloeckera</i> sp.2201	<i>Polyporus</i> sp.	<i>Poria contigua</i>	<i>Tanacetum vulgare</i>
METHANOL	100%	100%	100%	100%	100%	100%	0
ETHANOL	75%	50%	106%	106%	28%	94%	0
PROPAN-1-OL	25%	44%	79%	48%	5%	73%	0
BUTAN-1-OL	15%	22%	69%	31%	2%	-	1%
PENTAN-1-OL ( AMYL ALCOHOL )	5%	-	-	-	1%	-	2%
HEXAN-1-OL	0	-	-	1%	-	-	15%
PROPAN-2-OL	5%	-	-	3%	-	-	-
2-PROPEN-1-OL ( ALLYL ALCOHOL )	65%	49%	89%	67%	17%	-	7%
2-BUTEN-1-OL ( CROTYL ALCOHOL )	-	39%	73%	-	-	-	20%
HEX-TRANS-2-ENE-1-OL	-	-	-	-	-	-	100%
BENZYL ALCOHOL	-	-	-	0	0	-	0
REFERENCE	Sahm and Wagner(1973)	Kato <i>et al</i> (1976)	Kato <i>et al</i> (1976)	Tani <i>et al</i> (1972)	Janssen and Ruelius (1968)	Bringer <i>et al</i> (1979)	Banthorpe <i>et al</i> (1978)

**Table 1.3.5.b) Relative activity of several aliphatic alcohol oxidases with various substrates**  
 Activity is given as a percentage of the activity with methanol (yeasts and fungi) or with hex-trans-2-ene-1-ol (*Tanacetum vulgare*).  
 Activity with these substrates is arbitrarily defined as 100%.

for example methoxylated lignin subunits (Bringer *et al* 1979). The *Poria contigua* enzyme is localised in microbodies (peroxisomes) in the mycellium (Bringer *et al* 1980).

An alcohol oxidase has been reported in the leaves of the higher plant *Tanacetum vulgare* that differs considerably from yeast and fungal enzymes with respect to subunit composition and substrate specificity (see tables 1.3.5.a) and 1.3.5.b)) (Banthorpe *et al* 1976). Primary aliphatic alcohols of medium chain length (C<sub>6</sub> - C<sub>10</sub>) are good substrates as are  $\alpha$ - $\beta$ -unsaturated alcohols such as crotyl alcohol (but-2-ene-1-ol) and hex-*trans*-2-ene-1-ol (leaf alcohol). It is inactive with lower primary alcohols (methanol, ethanol etc.) and aromatic alcohols. Its physiological role is unknown.

An inducible long-chain (fatty) alcohol oxidase has been reported in alkane-grown *Candida tropicalis* (Kemp *et al* 1988). It is localised on the microsomal membranes and is distinct from short-chain alcohol (methanol) oxidase and NAD<sup>+</sup>-linked dehydrogenases (see section 1.3.3.). The enzyme is active with aliphatic alcohols of chain-length C<sub>6</sub> - C<sub>18</sub>; activity peaks with dodecan-1-ol (C<sub>12</sub>).

Another hydrogen peroxide generating fatty alcohol oxidase is found in the cotyledons of jojoba seedlings, *Simmondsia chinensis*. The enzyme is located in the membranes of the wax bodies. Activity peaks with dodecan-1-ol (C<sub>12</sub>) and has a pH optimum of 9.0. Fatty alcohols are derived from wax-esters and are physiological substrates in jojoba seedlings (Moreau and Huang 1979).

Extracellular soluble aromatic alcohol oxidases (often termed veratryl alcohol oxidase or VAO) are produced by a number of lignolytic white-rot fungi. This activity has been reported in *Bjerkandera adustus* (*Polyporus adusta*) (Muheim *et al* 1990), *Pleurotus sajor-caju* (Bourbonnais and Paice 1988), *Pleurotus eryngii* (Guillen *et al* 1990), *Pleurotus ostreatus* (Sannia *et al* 1991), *Coriolus versicolor* (*Polystictus versicolor*) (Farmer *et al* 1960) and *Fomes lignosus* (Waldner *et al* 1988). The reported activities show similar substrate preferences and are virtually inactive with aliphatic alcohols (table 1.3.5.c)). They are active across a broad pH range with optima



SUBSTRATE	SPECIES				
	<i>Coriolus versicolor</i>	<i>Bjerkandera adustus</i>	<i>Pleurotus ostreatus</i>	<i>Pleurotus eryngii</i>	<i>Pleurotus sajor-caju</i> VAO I VAO II
4-METHOXY-BENZYL ALCOHOL	100%	100%	100%	100%	100%
VERATRYL ALCOHOL	6%	8%	97%	64%	38%
CINNAMYL ALCOHOL	-	-	211%	79%	21%
CONIFERYL ALCOHOL	-	-	43%	0%	0%
BENZYL ALCOHOL	4%	5%	11%	17%	9%
3-METHOXY-4-HYDROXY-BENZYL ALCOHOL	12%	15%	24%	<1%	0%
4-METHOXY-3-HYDROXY-BENZYL ALCOHOL	-	-	140%	56%	27%
ALLYL ALCOHOL	-	-	6%	<1%	0%
Km FOR VERATRYL ALCOHOL	-	1.5mM	0.32mM	0.41mM	0.46mM
Km FOR 4-METHOXY-BENZYL ALCOHOL	-	0.24mM	-	0.04mM	-
pH OPTIMUM	6.0 - 6.8	5.7	6.5	6.0 - 6.5	5
REFERENCE	Farmer et al (1960)	Muheim et al (1990)	Sannia et al (1991)	Guillen et al (1990 & 1992)	Bourbonnais and Paice (1988)

**Table 1.3.5.c) Comparison of fungal aromatic alcohol oxidases**

Activity is expressed as a percentage of the activity with 4-methoxy-benzyl alcohol. Activity with 4-methoxy-benzyl alcohol is arbitrarily defined as 100%.

between pH 5.0 and 7.0. They are soluble flavoproteins of between 71kDa (*Bjerkandera adustus*) (Muheim *et al* 1990) and 78kDa (*Pleurotus sajor-caju*) (Bourbonnais and Paice 1988).

Aromatic alcohols are likely products of the enzymatic breakdown of lignin and could conceivably be oxidised by aromatic alcohol oxidase prior to further biodegradation. The hydrogen peroxide produced may be utilised by the extracellular enzyme responsible for lignin depolymerisation, lignin peroxidase, although hydrogen peroxide is also provided by other extracellular oxidases (for example glyoxal oxidase) in many species (Waldner *et al* 1988). The lignin peroxidase of *Phanerochaete chrysosporium* is capable of the peroxidatic oxidation of veratryl alcohol (to veratraldehyde) in the presence of hydrogen peroxide (Tien and Kirk 1978). This species does however lack aromatic alcohol oxidase activity.

In *Phanerochaete chrysosporium* veratryl alcohol is synthesised *de novo* from glucose (via L-phenylalanine) and its formation coincides with the onset of lignolytic activity (Lundquist and Kirk 1978). In several white-rot fungi veratryl alcohol is an inducer of the lignolytic system and can enhance the degradation of lignin. It has been suggested that veratryl alcohol is biosynthesised to protect lignin peroxidase from inactivation by hydrogen peroxide and to convert the inactivated enzyme back to its native form (DeJong *et al* 1994). Veratraldehyde produced by lignin peroxidase is converted back to veratryl alcohol by an aryl-aldehyde reductase (aryl-aldehyde NADPH oxidoreductase) and this helps to maintain the level of veratryl alcohol (Muheim *et al* 1991).

In *Bjerkandera* sp. strain B0S55 3-chloroanisyl and 3,5,-dichloroanisyl alcohols are synthesised *de novo* as well as veratryl alcohol. This fungus also produces extracellular aromatic alcohol oxidase (unlike *Phanerochaete chrysosporium*) which has a far greater affinity for the chloro-substituted anisyl alcohols (4-methoxy-benzyl alcohol) compared to veratryl alcohol. It is possible that the chloro-substituted anisyl alcohols are synthesised to protect veratryl alcohol from unwanted oxidation by aromatic alcohol oxidase and thereby maintain the level of veratryl alcohol which protects the lignolytic system. It is

worth noting that chloro-substituted anisyl alcohols are not peroxidatically oxidised by lignin peroxidase (DeJong *et al* 1994). The roles of aromatic alcohol oxidase and aromatic alcohols in lignin biodegradation are therefore complex and await further investigation.

An alcohol oxidase acting on  $\alpha$ - $\beta$ -unsaturated alcohol side-chains of lignin-related compounds has been reported in the fungus imperfecti *Fusarium solani*. This oxidase is specific for  $\alpha$ - $\beta$ -unsaturated primary alcohols (for example 3,4,-dimethoxy-cinnamyl alcohol) or the side-chains of dilignols (for example dehydroconiferyl alcohol) converting them to their corresponding aldehydes. The enzyme is inactive with aromatic alcohols such as veratryl, benzyl or anisyl alcohols (Iwahara 1984, Iwahara *et al* 1980).

A coniferyl alcohol oxidase has been reported in gymnosperm xylem (Savidge and Udagama-Randeniya 1992) but this catalyses the oxidative polymerisation (rather than oxidation to the corresponding aldehyde) of coniferyl alcohol to dilignols such as dehydroconiferyl alcohol and pinoresinol and produces water rather than hydrogen peroxide.

In conclusion alcohol oxidases would seem to be restricted to yeasts, fungi and a few higher plants. Alcohol oxidation in animals is mainly mediated by NAD<sup>+</sup>-linked dehydrogenases. This thesis describes an alcohol oxidase with a preference for aromatic alcohols in the tissues of gastropod molluscs, an activity that appears to be unique in the animal kingdom. Aromatic alcohol oxidase has previously been considered to be an activity restricted to a few lignolytic basidiomycete fungi.



### 1 NAD(P)<sup>+</sup>-dependent Dehydrogenases



### 2 NAD(P)<sup>+</sup>-independent (PMS-linked) Dehydrogenases



### 3 Oxidases



**Figure 1.4. A summary of the reactions of various aldehyde oxidising enzymes**

Cyt = cytochrome  
(ox) = oxidised form  
(red) = reduced form

## 1.4. Aldehyde Oxidation

Aliphatic and aromatic aldehydes are frequently encountered as physiological substrates. Aldehydes are the products of the alcohol oxidising enzymes described in the previous section. They are also the products of mono-amine oxidases and microbial amine dehydrogenases. In mammals aldehyde dehydrogenases are studied with respect to their role in ethanol metabolism whereas mammalian aldehyde oxidase is studied with respect to its activity with purines and purine analogues rather than with aldehydes. Aldehyde oxidising enzymes play an important role in the biodegradation of alkanes and methane by alkane-utilising and methylotrophic micro-organisms. Long-chain unsaturated aliphatic aldehydes are pheromone components in several species of insects and specific enzymes are responsible for their oxidative degradation.

Aldehyde oxidising enzymes can be broadly categorised into three groups :- 1) NAD(P)<sup>+</sup>-dependent dehydrogenases, 2) NAD(P)<sup>+</sup>-independent dehydrogenases and 3) hydrogen peroxide producing aldehyde oxidases (see figure 1.4.). Each group will be considered in turn.

### 1.4.1. NAD(P)<sup>+</sup>-Dependent Aldehyde Dehydrogenases

NAD(P)<sup>+</sup>-linked aldehyde dehydrogenases are widely distributed in vertebrates, invertebrates, plants, fungi and micro-organisms. Mammalian aldehyde dehydrogenases (aldehyde : NAD<sup>+</sup> oxidoreductase) vary between species but in general are tetrameric proteins with subunits of between 48kDa and 57kDa. They will oxidise a variety of aliphatic and aromatic substrates but their activity with acetaldehyde (ethanal) is significant because it is an intermediate in ethanol metabolism.

The subcellular localisation and number of iso-enzymes also varies between species. Sheep and horse liver both contain two iso-enzymes (one cytosolic and one mitochondrial). The iso-enzymes in the same species vary with respect to the  $K_m$  values for  $NAD^+$  and acetaldehyde (Crow *et al* 1974, Eckfeldt *et al* 1976). Human liver also has two iso-enzymes of differing kinetic parameters (Greenfield and Pietruszko 1977). Rat liver normally contains at least four iso-enzymes distributed in the mitochondria and microsomes. A further four iso-enzymes are inducible in this tissue by drugs or by tumours (Lindahl and Evacs 1984a,b). A peroxisomal aldehyde dehydrogenase is also inducible by clofibrate in rat liver (Panchenko *et al* 1982).

Bakers yeast (*Saccharomyces cerevisiae*) has a potassium activated aldehyde dehydrogenase that is also tetrameric (sub-unit size 62.5kDa) and oxidises a variety of aliphatic and aromatic substrates (Bostian and Betts 1978). A more specific aldehyde dehydrogenase is found in alkane-grown yeasts (for example *Candida* species) that is most active with long-chain ( $C_7 - C_{14}$ ) aliphatic aldehydes. Alkanes are initially hydroxylated to alcohols and the concerted action of alcohol and aldehyde dehydrogenases specific for long-chain substrates convert these alcohols to fatty acids. These are then assimilated into lipids or utilised via peroxisomal  $\beta$ -oxidation (Ueda and Tanaka 1990). A long-chain aldehyde dehydrogenase has been reported in the cotyledons of jojoba seedlings (Moreau and Huang 1979). This activity is localised in the membrane of the wax-bodies together with a long-chain alcohol oxidase (see section 1.3.5.). The concerted action of these two activities converts fatty alcohol derived from the hydrolysis of stored wax esters into fatty acids.

Methylotrophic yeasts (*Candida boidinii*, *Hansenula polymorpha*, *Kloeckera* sp.) have a highly specialised  $NAD^+$ -linked formaldehyde dehydrogenase. The activity is dependent on the presence of glutathione as the actual substrate is *S*-hydroxymethyl-glutathione (formed spontaneously from formaldehyde and glutathione). The product of the reaction is *S*-formyl-glutathione (Kato 1990).



There are very many bacterial NAD<sup>+</sup>-linked aldehyde dehydrogenases. For example *Proteus vulgaris* has an aldehyde dehydrogenase that is most active with short-chain aliphatic aldehydes and is NADP<sup>+</sup>-specific (Sugawara and Sasaki 1977). Two soluble broad-range aldehyde dehydrogenases are found in *Pseudomonas aeruginosa* one of which is NAD<sup>+</sup>-specific and one NADP<sup>+</sup>-specific (Guerrillot and Vandecasteele 1977). Benzaldehyde dehydrogenase activity is found in several bacteria including *Acinetobacter calcoaceticus*. The non-inducible benzaldehyde dehydrogenase I activity is normally very low however when the bacteria is grown with benzyl alcohol as the sole carbon source a more active benzaldehyde dehydrogenase II is induced. This benzaldehyde dehydrogenase II is a tetrameric protein of 225kDa (subunits of 55kDa) in common with mammalian aldehyde dehydrogenase (MacIntosh and Fewson 1988). Bacterial formaldehyde dehydrogenases (NAD<sup>+</sup>-linked) (for example as found in *Methylophilus methylotrophus*, *Arthrobacter* P1) differ from yeast formaldehyde dehydrogenases in that formaldehyde rather than S-hydroxymethyl-glutathione is the substrate (Attwood 1990).

The above is not an exhaustive list of aldehyde dehydrogenases but is representative of a very widely distributed and variable type of activity.

#### 1.4.2. NAD(P)<sup>+</sup>-independent Aldehyde Dehydrogenases

There is a group of aldehyde dehydrogenases that are NAD(P)<sup>+</sup>-independent but that can utilise a variety of artificial electron acceptors such as phenazine methosulphate (PMS). This type of activity is analogous to the NAD(P)<sup>+</sup>-independent alcohol dehydrogenases describes previously (see section 1.3.4.). Aldehyde dehydrogenase from the bacteria *Hyphomicrobium* X is one such enzyme. It oxidises a wide variety of aldehydes including formaldehyde (Attwood 1990).

Acetic acid bacteria (*Acetobacter aceti* and *Gluconobacter suboxydans*) also have NAD(P)<sup>+</sup>-independent aldehyde dehydrogenases that contain pyrroloquinoline quinone (PQQ) much

like the corresponding alcohol dehydrogenases in these species (see table 1.3.4.). The enzyme from both species is active with many aldehydes but not with formaldehyde. Aldehyde dehydrogenase is responsible for the conversion of acetaldehyde to acetic acid and is localised on the surface of the cytoplasmic membrane where it forms a multienzyme complex with alcohol dehydrogenase. Both alcohol and aldehyde dehydrogenase are linked to the respiratory chain via the cytochrome component of the enzyme (Ameyama and Adachi 1982).

### 1.4.3. Aldehyde Oxidases

Aldehyde oxidases (aldehyde : oxygen oxidoreductase) are enzymes that oxidise aldehyde to their corresponding carboxylic acid and produce hydrogen peroxide by the partial reduction of molecular oxygen. Mammalian aldehyde oxidase has been thoroughly characterised. Aldehyde oxidase activity is however found throughout the animal kingdom (Wurtzinger and Hartenstein 1974).

Mammalian hepatic aldehyde oxidase is a flavoprotein that contains molybdenum and a quinone (co-enzyme Q<sub>10</sub>). The bovine hepatic enzyme has a native molecular weight of 260 - 270 kDa and has five non-identical subunits. Xanthine oxidase from the same source is a clearly related enzyme but does not contain a quinone and has one less subunit (Cabre and Canela 1987). Although both mammalian aldehyde and xanthine oxidases catalyse the oxidation of many aliphatic and aromatic aldehydes *in vitro* this does not appear to be their function *in vivo*. Aldehyde oxidation is mostly catalysed by NAD<sup>+</sup>-linked dehydrogenases in mammalian tissues which have a greater affinity for aldehydes (Krenitsky *et al* 1972).

Cytosolic aldehyde oxidase and xanthine oxidase are capable of the oxidation of a broad range of purines, pyrimidines and xenobiotic purine analogues. The activity of these two enzymes is also complementary : a purine that is a poor substrate for one enzyme will

often prove to be more actively oxidised by the other enzyme. These two enzymes therefore represent a pair of substituted purine/pyrimidine oxidases and form a detoxification system for the removal of endogenously produced or ingested purines, pyrimidines and xenobiotic purine analogues. In many studies 6-methyl-purine and N<sup>1</sup>-methylnicotinamide are used as the substrates for the estimation of aldehyde oxidase activity as these are highly specific for mammalian aldehyde oxidase. Xanthine oxidase is virtually inactive with these substrates (Krenitsky *et al* 1972, Hall and Krenitsky 1986).

These two activities have aroused interest because they may be important in the metabolism of some heterocyclic anti-cancer agents. Many anti-cancer drugs or their metabolites (such as the metabolites of 6-mercapto-purine) are effective by interfering in nucleotide metabolism and therefore cell division (Stet *et al* 1991). Some of these anti-cancer drugs are purines or purine analogues and are potential substrates for aldehyde oxidase and xanthine oxidase; for example 6-mercapto-purine is a substrate for both enzymes (Krenitsky *et al* 1972).

Invertebrate aldehyde oxidases are far less well characterised. In one study (Wurzinger and Hartenstein 1986) some seventy-nine animal species were surveyed for aldehyde oxidase, xanthine oxidase and xanthine dehydrogenase. Vanillin (4-hydroxy-3-methoxy-benzaldehyde) was chosen as the substrate for aldehyde oxidase and hypoxanthine as the substrate for xanthine oxidase and dehydrogenase. It was found that aldehyde oxidase makes its first phylogenetic appearance in molluscs and is found in most phylogenetically less primitive species. Xanthine dehydrogenase appears first in insects and is found in many less primitive species. Xanthine oxidase appears to be limited to birds and mammals only.

Aldehyde oxidase has been studied in *Drosophila melanogaster* and is similar in several ways to the mammalian enzyme with a native molecular weight of 280kDa and a similar FAD/molybdenum content (Andres 1976). An aldehyde oxidase has also been described in the crayfish *Cambarus bartoni* (Hartenstein 1973). Although active with a variety of aldehydes the enzyme is inactive with N<sup>1</sup>-methylnicotinamide



(a substrate for mammalian aldehyde oxidase). The native molecular weight is in excess of 200kDa.

Specialised aldehyde oxidase activities are found in the sensory-organs of the moths *Manduca sexta* (Rybczynski *et al* 1989) and *Heliothis virescens* (Tasayco and Prestwich 1990). These activities are distinct from the other aldehyde oxidising enzymes found in the tissues of these insects. They show a high affinity for the pheromone components (Z)-9-tetradecanal, (Z)-11-hexadecanal (*H. virescens*) and bombykal ((E,Z)-10,12-hexadecadienal) (*M.sexta*). They are also active with several aldehydes including benzaldehyde and hex-*trans*-2-enal (an aldehyde commonly found in leaves). It is possible that these enzymes play a modulating role in the sensitivity of adult moths to aldehyde odours and also degrade aldehyde odours in general(Rybczynski *et al* 1989).

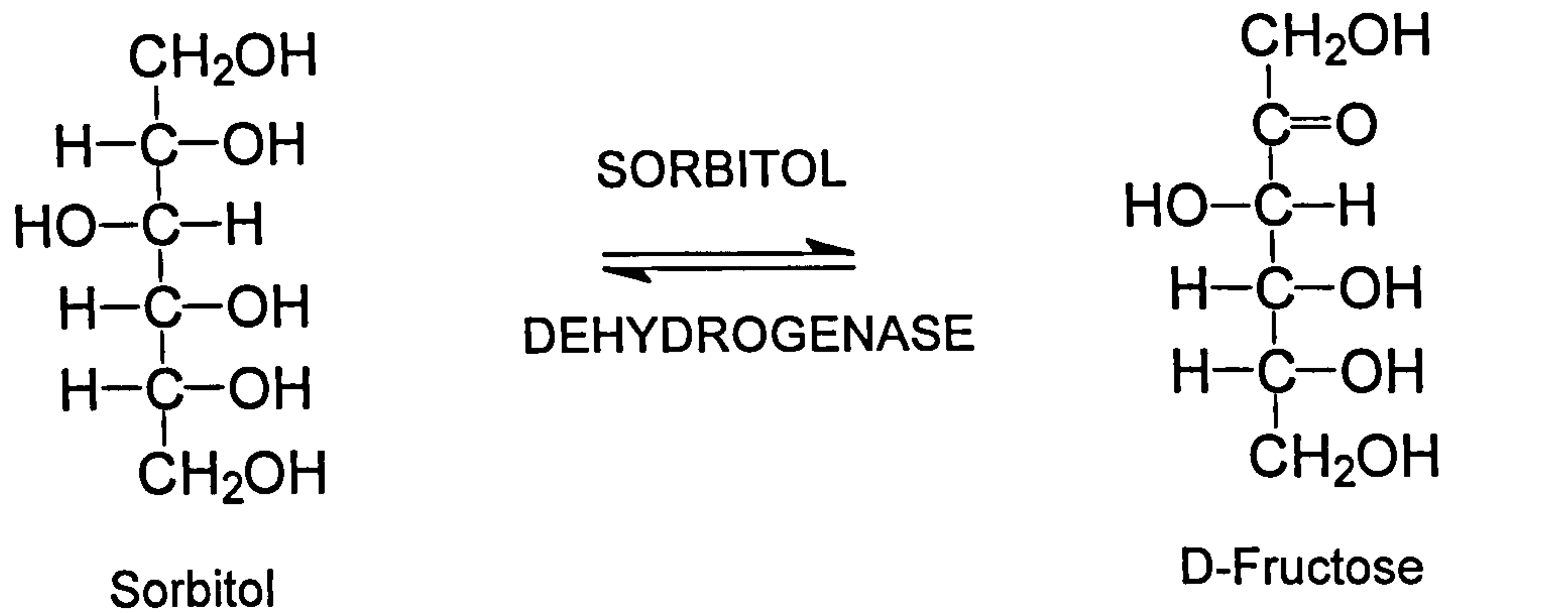
This thesis describes the aldehyde oxidase activities found in the tissues of several terrestrial gastropods. This activity has been studied because of its possible role in the oxidation of the products of the aromatic alcohol oxidase also found in these animals. Detoxification and digestive roles have been considered for this activity.

## 1.5. Sugar Alcohol Oxidation

Sugar alcohols are the reduction products of sugars (aldoses and ketoses) and are a type of polyol. Enzymes that act on sugar alcohols will be considered here; enzymes that act on other types of polyol (for example *myo*-inositol) and glycerol will not be considered.

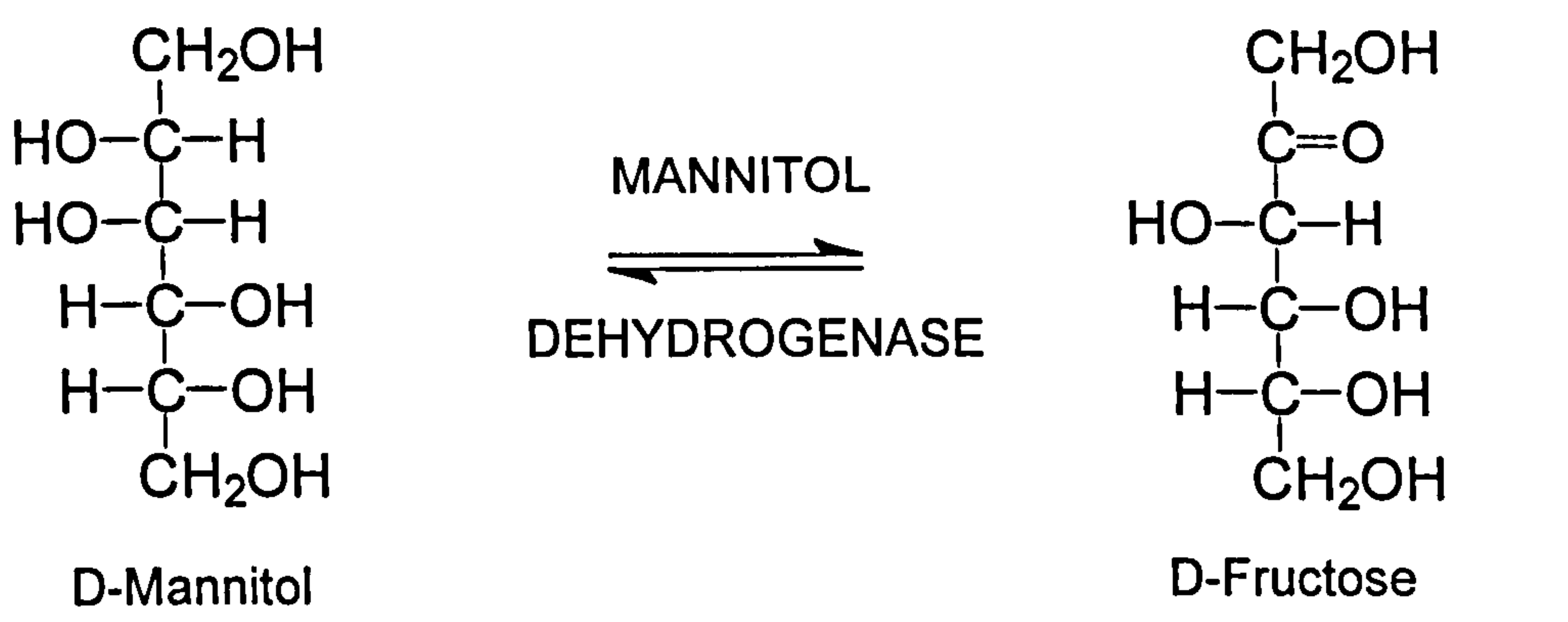
Sugar alcohols are found widely in nature particularly in plants, fungi and algae. In many fungi D-mannitol is an important storage carbohydrate and may form up to 13% of the dry weight of the organism. Fungi may also contain other sugar alcohols. Sugar alcohols including D-mannitol are found in algae (particularly marine algae) and again form a high percentage of the dry weight. In higher plants there are also examples of significant sugar alcohols content. Sorbitol (D-glucitol) is found in plants belonging to the *Rosaceae* family. Other families also contain various sugar alcohols; for example members of the *Oleaceae* family contain D-mannitol and this may form up to 8% of the dry weight. D-Ribitol is found in the sap of members of the *Meliceae* and *Rutaceae* families. Sugar alcohols may function as storage products and transport intermediates (as they are non-reducing unlike aldoses and ketoses). They may also play a role as the intermediates in the interconversion of sugars. Osmoregulation and anti-freezing have also been suggested as roles for these compounds (Lewis and Smith 1967).

Therefore sugar alcohols are abundant carbohydrates. Utilisation by bacteria or herbivores would normally involve oxidation to their corresponding ketose or aldose. Degradation would then be via, for example, glycolysis or the pentose-phosphate pathway. However enzymes that oxidise sugar alcohols may not play any role in digestion at all but rather function in the interconversion of sugars, for example the sorbitol dehydrogenase of mammalian lens (Walsall *et al* 1978).



+ NAD(P)<sup>+</sup>

+ NAD(P)H + H<sup>+</sup>



+ NAD(P)<sup>+</sup>

+ NAD(P)H + H<sup>+</sup>

Figure 1.5.1.1. The reactions of mannitol dehydrogenase and sorbitol dehydrogenase



Most of the enzymes that oxidise sugar alcohols and sugar alcohol-phosphates are NAD(P)<sup>+</sup>-linked dehydrogenases and such enzymes are found in micro-organisms, plants and animals. There is also a report of a cytochrome-linked NAD(P)<sup>+</sup>-independent sorbitol dehydrogenase in the bacteria *Gluconobacter suboxydans* (Kerstens *et al* 1965). Sugar alcohol oxidases are very unusual activities and thus far appear to be restricted to plant leaves (water-producing sorbitol oxidase) and gastropod molluscs (hydrogen peroxide-generating D-mannitol oxidase). Each type of activity will be considered in turn.

### 1.5.1. NAD(P)<sup>+</sup>-dependent Sugar alcohol Dehydrogenases

#### 1.5.1.1. Hexitol Dehydrogenases

Hexitols, including D-mannitol and sorbitol, are oxidised by a number of NAD(P)<sup>+</sup>-linked enzymes in animals and micro-organisms. The product of sorbitol and D-mannitol oxidation is D-fructose (see figure 1.5.1.1.). Mammalian sorbitol dehydrogenase is a member of the zinc-containing long-(polypeptide)-chain alcohol dehydrogenase family of enzymes (see section 1.3.3.). It is a tetrameric protein of 140kDa (40kDa subunits) (Jörnvall *et al* 1987). Hepatic sorbitol dehydrogenase is active with hexitols (sorbitol and D-mannitol) and pentitols (D-ribitol and D-xylitol) but is not active with sugar alcohol phosphates. Substrate specificity varies between species but sorbitol is always a good substrate. The hepatic enzyme probably oxidises exogenous sugar alcohol substrates *in vivo*. Sorbitol dehydrogenase activity is found in several other mammalian tissues including lens, nerve and seminal vesicles. The enzyme is probably involved in hexose interconversions in these tissues; D-glucose is reduced by aldose reductase to give sorbitol which is then oxidised to D-fructose by sorbitol dehydrogenase. The action of these two enzymes in the

seminal vesicles probably accounts for the fructose content of seminal fluid (Walsall *et al* 1978).

Sorbitol dehydrogenase and D-mannitol dehydrogenase activities are also found in fungi and bacteria. Examples of bacterial activities include D-mannitol dehydrogenase of *Lactobacillus brevis* (Martinez *et al* 1963) and sorbitol dehydrogenase of *Bacillus subtilis* (Horowitz and Kaplan 1964). Examples of fungal sugar alcohol dehydrogenases include D-mannitol dehydrogenase of *Absidia glauca* (Ueng *et al* 1976); the D-mannitol dehydrogenase of *Aspergillus parasiticus* is unusual in being NADP<sup>+</sup>-specific rather than NAD<sup>+</sup>-specific (Niehaus and Dilts 1982).

#### 1.5.1.2. Hexitol-phosphate Dehydrogenases

In many bacteria catabolism of D-hexitols involves two hexitol-specific steps. The first step is phosphorylation of the hexitol coupled to trans-membrane transport. The second step is the oxidation of the hexitol-phosphate by specific cytoplasmic dehydrogenases to the corresponding ketose-phosphate (Novotny *et al* 1984). In *Escherichia coli* there are two distinct and specific hexitol-phosphate dehydrogenases. Sorbitol-6-phosphate (D-glucitol-6-phosphate) dehydrogenase is a member of the short-(polypeptide)-chain alcohol dehydrogenase family of enzymes (see section 1.3.3.) (Yamada and Saier 1987). It is a tetrameric protein of 117kDa (26kDa subunits). D-Mannitol-1-phosphate dehydrogenase from the same source is not a member of this family of enzymes and is a monomeric protein of 40kDa (Novotny *et al* 1984). D-Mannitol-1-phosphate dehydrogenase from *E.coli* has been reported as being a dimeric protein of 44kDa (22kDa subunits) (Chase 1986).

Examples of other bacterial activities include sorbitol-6-phosphate and D-mannitol-1-phosphate dehydrogenases of *Aerobacter (Klebsiella) aerogenes* (Liss *et al* 1962) and D-mannitol-1-phosphate dehydrogenase of *Bacillus subtilis* (Horowitz and Kaplan 1964). D-Mannitol-1-phosphate dehydrogenase activity is also found in fungi

including *Aspergillus niger* and *Aspergillus parasiticus*. This specific enzyme is a dimeric protein of 44kDa (22kDa subunits)(Foreman and Niehaus 1985). All of the above enzymes produce the glycolytic intermediate D-fructose-6-phosphate from both D-mannitol-1-phosphate and sorbitol-6-phosphate.

D-Galactitol-6-phosphate dehydrogenase is an unusual specific and inducible activity found in the bacterium *Klebsiella pneumoniae*. The product is D-tagatose-6-phosphate, a ketose phosphate (Anderson and Markwell 1982).

### 1.5.1.3. Pentitol Dehydrogenases

The most studied pentitol dehydrogenase is the D-ribitol dehydrogenase of the bacterium *Klebsiella aerogenes* which is a member of the short-(polypeptide)-chain alcohol dehydrogenase family of enzymes (see section 1.3.3.)(Persson *et al* 1991). It is a tetrameric protein of four identical 27kDa subunits (Taylor *et al* 1974). D-Ribitol dehydrogenase is specific for D-ribitol with only limited activity with D-xylitol. However a mutant strain of *Klebsiella aerogenes* produces a D-ribitol dehydrogenase that shows a greater affinity for D-xylitol than the normal enzyme (Loviny *et al* 1985). A distinct D-arabitol dehydrogenase is also found in this species of bacterium. The product of the pentitol dehydrogenases described above is D-xylulose from D-ribitol, D-xylitol and D-arabitol. D-Xylulose (a ketose) is phosphorylated by D-xylulokinase to produce D-xylulose-5-phosphate which is an intermediate of the pentose phosphate pathway (Loviny *et al* 1985, Fossit and Wood 1966). The phototrophic bacterium *Rhodobacter sphaeroides* has an inducible D-ribitol dehydrogenase that is a dimeric protein of 50kDa (25kDa subunits) (Kahla *et al* 1992).

The D-xylitol dehydrogenase of *Penicillium chrysogenum* is an example of a fungal pentitol dehydrogenase. This enzyme acts together with xylose reductase to interconvert pentoses. Xylose reductase reduces xylose to D-xylitol; D-xylitol is then oxidised to D-xylulose by the dehydrogenase (Chiang and Knight 1966).



### 1.5.2. NAD(P)<sup>+</sup>-independent Sugar Alcohol Dehydrogenases

The acetic acid bacterium *Gluconobacter suboxydans* has a NAD(P)<sup>+</sup>-independent sorbitol dehydrogenase. The activity is particulate and located on the cytoplasmic membrane. The electron acceptor *in vivo* is a cytochrome which is linked to the respiratory chain; this activity is therefore similar to the NAD(P)<sup>+</sup>-independent alcohol and aldehyde dehydrogenases in acetic acid bacteria (see table 1.3.4. and sections 1.3.4. and 1.4.2.). The enzyme may utilise several artificial electron acceptors *in vitro* including phenazine methosulphate. Sorbitol is oxidised to L-sorbose (a ketose). D-Mannitol is oxidised at 5% the rate of sorbitol and the product of D-mannitol oxidation is D-fructose (Kerstens et al 1965, Shinagawa et al 1982).

### 1.5.3. Sugar Alcohol Oxidases

Sugar alcohol oxidases have rarely been reported. There are just two reports of such activities :- water-producing sorbitol oxidase in apple leaves and hydrogen peroxide-generating D-mannitol oxidase in terrestrial gastropod molluscs.

Sorbitol is a major soluble carbohydrate in the leaves of many species of the *Rosaceae* family of plants and is also an important transport-intermediate in the phloem. Sorbitol dehydrogenase (NAD<sup>+</sup>-linked) in the tissues of apple trees catalyses the conversion of sorbitol to D-fructose. However in the leaves an enzyme activity is found which is very different from the sorbitol dehydrogenase encountered in plants, animals and micro-organisms. Apple-leaf sorbitol oxidase is NAD(P)<sup>+</sup>-independent and utilises molecular oxygen as the electron acceptor; oxygen is fully reduced to water. This activity is also unlike cytochrome-linked sorbitol dehydrogenase (see section 1.5.2.) as it is not inhibited by cyanide and does not contain a cytochrome



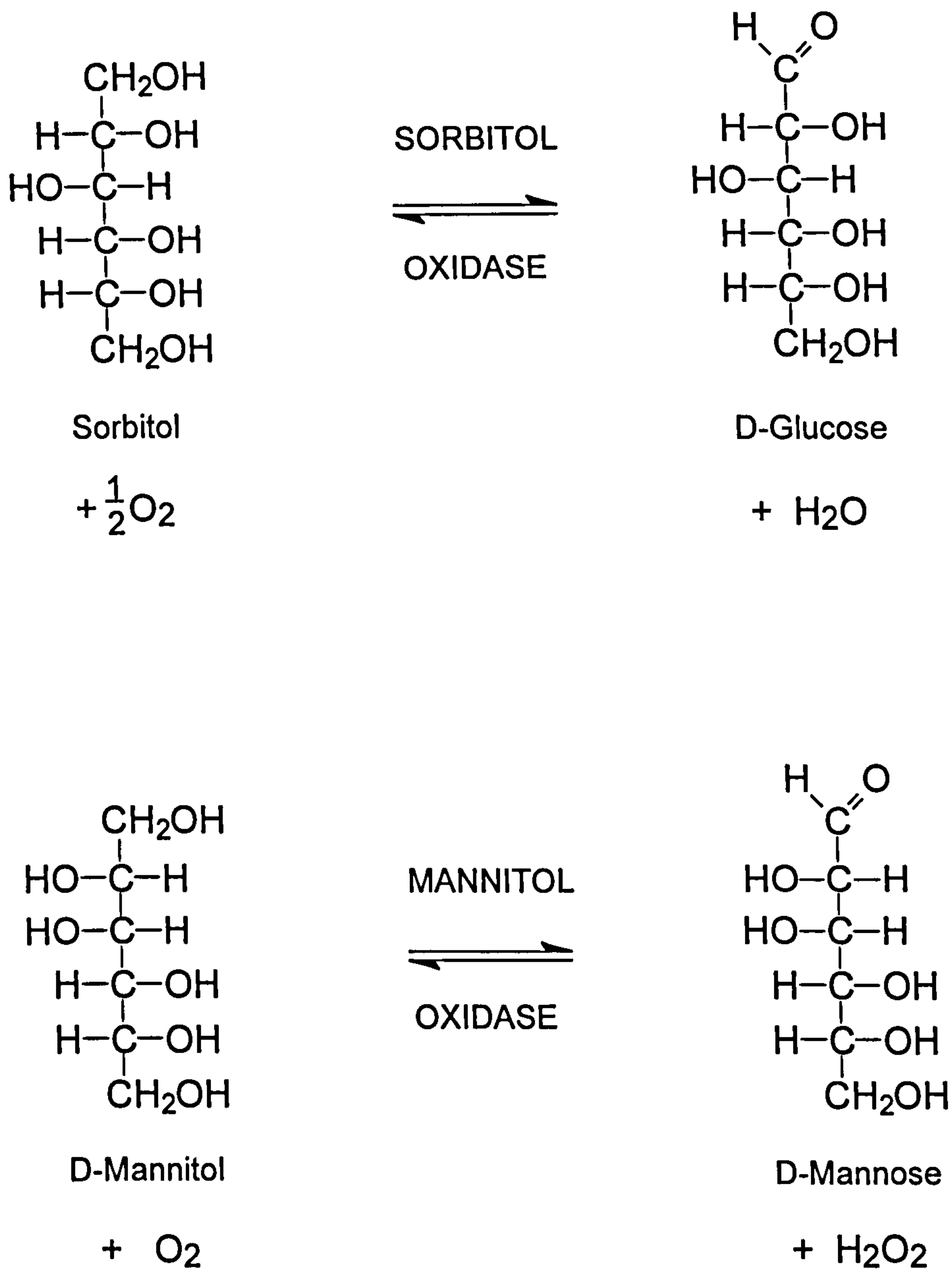


Figure 1.5.3. The reactions of mannitol oxidase and sorbitol oxidase

component. Significantly the product is D-glucose (an aldose)(see figure 1.5.3.) rather than D-fructose or L-sorbose (ketoses and the products of NAD<sup>+</sup>-linked and NAD(P)<sup>+</sup>-independent sorbitol dehydrogenases respectively). The activity has a pH optimum of 4.0 and an apparent  $K_m$  value of 100mM for sorbitol at this pH. The physiological function appears to be the interconversion of hexoses (Yamaki 1980).

D-Mannitol oxidase activity was first identified in digestive gland mitochondrial membrane-enriched fractions of the garden snail *Helix aspersa* (Vorhaben *et al* 1980). Mannitol-dependent respiration in the mitochondrial fractions was found to be cyanide-insensitive. The oxidation of D-mannitol is NAD(P)<sup>+</sup>-independent and the product of oxidation is D-mannose (an aldose) rather than D-fructose (see figure 1.5.3.). Hydrogen peroxide is also produced in stoichiometric quantities. It is possible that D-mannitol utilisation would be via mannitol oxidase, hexokinase and phosphomannose isomerase to produce D-glucose-6-phosphate, a glycolytic intermediate. The more usual first-step in the pathway of D-mannitol and sorbitol utilisation would be oxidation by a hexitol dehydrogenase to D-fructose (see section 1.5.1.1.)

Later mannitol oxidase was shown to be localised in the wall of the alimentary canal and in the digestive gland (hepatopancreas) of *Helix aspersa* (Vorhaben *et al* 1984). This localisation of the activity strongly suggests a significant role in the utilisation of dietary D-mannitol and that molluscs derive nutritional benefit from ingested mannitol. Vorhaben *et al* (1986) solubilised and partially purified the enzyme (250-fold compared to the tissue homogenate). They found a stoichiometry of 1.0 mannose to 0.86 hydrogen peroxide produced. D-Mannitol ( $K_m$  5.9 - 6.9 mM) and D-arabitol are the most favoured substrates but sorbitol (D-glucitol) and dulcitol (D-galactitol) are also oxidised to a lesser extent. D-Ribitol, D-xylitol, D-erythritol and glycerol are not substrates. The enzyme has a pH optimum of 8.0 - 8.5. S.D.S.-P.A.G.E. analysis of the 250-fold purified preparation showed a predominant band at 68kDa as well as higher molecular weight proteins that may be contaminants. There is no evidence of flavin or cytochrome co-factors.

Mannitol oxidase has also been reported in the slug *Arion ater* (Malik *et al* 1987). Sucrose density-gradient centrifugation showed that mannitol oxidase is localised in a low-density membrane structure (density = 1.15 g cm<sup>-3</sup>) which is distinct from mitochondria or peroxisomes. It appears that molluscan D-mannitol oxidase is a unique dietary adaption to a herbivorous diet. This thesis describes the localisation of D-mannitol oxidase to a distinct tubular organelle in *Arion ater*. The sedimentation properties of D-mannitol oxidase bearing membranes has been investigated in several species of gastropod. The substrate specificity of the enzyme has also been compared in several species.

## 2. MATERIALS AND METHODS



## 2.1. Animals

Large specimens of *Arion ater*, *Arion luscitanicus*, *Limax flavus* and *Helix aspersa* were collected from local urban gardens. *Helix aspersa* was also obtained from a commercial supplier. The animals were either used immediately or maintained in containers on damp tissue and fed cucumber slices. Slug species were identified using *A Field Guide to the Slug Species of the British Isles* (Cameron *et al* 1983).

Large adult specimens of *Achatina fulica* (90 - 130g) were obtained from a commercial supplier and maintained in a vivarium at 22°C. They were fed on either cucumber, moistened porridge oats or a commercial powdered mollusc diet.

## 2.2. Subcellular Fractionation

### 2.2.1. Preparation of Homogenates

Digestive gland was dissected from decapitated animals and diced on an ice-cold vitreous surface with a razor blade. The diced tissue was gently homogenised using a Potter-Elvehjem type homogeniser in either 8.5% w/w sucrose or 10% w/w sucrose containing 3mM imidazole pH 7.2. The homogenate was subjected to low speed centrifugation ( 2,000g for 2.5 minutes ) to remove nuclei and cell debris; the supernatant was designated as the "Post Nuclear Supernatant" (hereafter referred to as P.N.S.). The volume was adjusted using 8.5% or 10% w/w sucrose according to the quantity of tissue used and the gradient onto which the P.N.S. was to be loaded.

### 2.2.2. Sucrose Density Gradient Centrifugation Methods

#### 2.2.2.1. Isopycnic Gradient Method Using the BXIV Rotor with the B29 Insert - Method for *Arion ater*

The following reproducible method was developed for density-dependent banding experiments using *Arion ater* digestive gland P.N.S.. A Beckman BXIV zonal rotor was used with a B29 insert to allow peripheral loading. The loading of the rotor peripherally caused air to be displaced from the central feed-line. Whilst the rotor was spinning at 2,000 r.p.m. it was loaded peripherally in the order as follows :- 50ml of 8.5% w/w sucrose overlay, 100ml of digestive gland P.N.S. (prepared in 10% w/w sucrose) and 30ml

each of 13, 15 , 20, 25, 30, 35, 40, 45, 50, 55 and 60% w/w sucrose (all containing 3mM imidazole pH 7.2). Centrifugation was at 45,000 r.p.m. for 2.5 hours to allow the organelles to equilibrate to their respective densities.

The rotor was unloaded centrally whilst spinning at 2,000 r.p.m. by displacing the contents with Fluorochemical FC43 (density = 1.9 g/ml) pumped in peripherally. A large volume fraction (200ml) was unloaded first which corresponded to the overlay, sample and the 13% w/w run-in. Thereafter fractions of 20ml were unloaded until finally Fluorochemical had displaced the rotor contents.

#### 2.2.2.2. Isopycnic Gradient Method Using the BXIV Rotor with the B29 Insert - Method for *Achatina fulica*

For density-dependent banding experiments with *Achatina fulica* digestive gland P.N.S. the Beckman BXIV rotor with a B29 insert was again employed. The method was essentially the same as described for *Arion ater* although the volumes were changed for the loading of the gradient and the unloading of the fractions. Approximately 165ml of 7.5% w/w sucrose overlay was loaded peripherally followed by 200ml of digestive gland P.N.S. (prepared as described previously in 8.5% w/w sucrose) and 27ml each of 10, 20, 30, 40 and 50% w/w sucrose. This was followed by sufficient 60% w/w sucrose to fill the rotor (~30ml). All sucrose solutions contained 3mM imidazole pH 7.2. Centrifugation was at 45,000 r.p.m. for 2.5 hours to allow the organelles to equilibrate to their respective volumes. The rotor was unloaded by peripheral displacement with Fluorochemical FC43 (density = 1.9 g/ml). A large volume fraction of 360ml was collected first corresponding roughly to the sample and overlay. Then a series of 16ml fractions corresponding to the gradient was unloaded until the Fluorochemical had displaced the rotor contents.

### 2.2.2.3. Rate-Dependent Sedimentation in a Sucrose Gradient Using the BXIV Rotor - Method for *Arion ater*

For rate-dependent banding experiments with *Arion ater* digestive gland P.N.S. a Beckman or M.S.E. BXIV rotor was employed. After several trials the following method was found to give the best separation of the mannitol oxidase bearing membranes from other organelles. A 500ml continuous gradient of 12 -28% w/w sucrose (containing 3mM imidazole pH 7.2) was loaded peripherally whilst the rotor was running at 2,000 r.p.m. (Beckman) or 3,000 r.p.m. (M.S.E.). The gradient volume was followed by sufficient 50% w/w sucrose (~150ml) to completely fill the rotor. 28ml of P.N.S. (prepared as described previously in 8.5% w/w sucrose) was loaded through the central feed-line followed by 27ml of 7% w/w sucrose overlay. This displaced 55ml of the 50% w/w sucrose from the peripheral feedline. Centrifugation was at 16,000 r.p.m. for exactly 30 minutes. After this time had elapsed the rotor was slowed to 2,000 r.p.m. (Beckman) or 3,000 r.p.m. (M.S.E.) and unloaded by peripheral displacement with Fluorochemical FC43. A fraction of 55ml was unloaded first (corresponding to the sample and overlay) followed by approximately twenty 30ml fractions.

### 2.2.2.4. Dual Gradient Method for the Enrichment of Mannitol Oxidase Bearing Membranes - *Arion ater*

The membranes associated with mannitol oxidase activity could be isolated in higher purity by subjecting *Arion ater* digestive gland P.N.S. to two consecutive sucrose gradient centrifugations. Digestive gland P.N.S. was prepared from several large specimens of *Arion ater* in a minimum volume of 8.5% w/w sucrose as described previously. 27ml of this P.N.S. was subjected to rate - dependent sedimentation as described above. The peak mannitol oxidase fractions were pooled and then loaded onto an isopycnic gradient in the Beckman BXIV rotor with a B29 insert. 30ml each of 8.5, 10, 11.5, 13 and 15% w/w sucrose (all containing 3mM



imidazole pH 7.2) were loaded peripherally followed by approximately 100ml of mannitol oxidase enriched sample from the rate gradient (with a sucrose concentration of roughly 20% w/w). This was followed by 30ml each of 25, 30, 35, 40, 45 and 50% w/w sucrose, 70ml of 55%w/w sucrose and finally 30ml of 60% w/w sucrose (all containing 3mM imidazole pH7.2). Centrifugation was at 45,000 r.p.m. for 2 hours. The rotor was then slowed to 2,000 r.p.m. and unloaded by peripheral displacement with Fluorochemical FC43 (density = 1.9 g/ml). The first fraction unloaded corresponded to the overlay and the sample and had a volume of 230ml. Thereafter ten 20ml fractions were unloaded followed by a large volume fraction (~100ml) corresponding to the 55%/60% w/w sucrose cushion. Peak mannitol oxidase fractions from this gradient were used for electron microscopic examination and S.D.S.-P.A.G.E. analysis.

#### 2.2.2.5. Isopycnic Gradient Method Using the Beckman Ti 50 Vertical Rotor

Multiple small-scale density-dependent banding experiments could be rapidly performed using the Beckman Ti 50 vertical rotor. P.N.S. was prepared from digestive gland in 8.5% w/w sucrose as described previously although care was taken to keep the volume to a minimum to ensure the P.N.S. was sufficiently concentrated (approximately one digestive gland from a large individual homogenised in ~10ml of 8.5% w/w sucrose was found to be satisfactory). P.N.S. (2ml) was loaded onto a gradient (which had been allowed to equilibrate overnight) consisting of 6ml volumes of 60, 50, 40, 30, 20 and 10% w/w sucrose (all containing 3mM imidazole pH 7.2) held in Beckman "quick-seal" tube. An overlay of approximately 3ml 7% w/w sucrose was added above the P.N.S. sample to completely fill the tube which was then heat-sealed. Centrifugation was at 45,000 r.p.m. ( $g_{\max} = 196,000\text{ g}$ ) for 2 hours in the Ti-50 rotor. It was important to use the minimum acceleration and deceleration profile settings on the centrifuge to ensure that the gradient was not disrupted.

When the centrifugation was complete the top of the tube was cut off and the bottom pierced with a hollow needle. The contents were unloaded by upward displacement with Fluorochemical FC43 using the Beckman apparatus for unloading "quick-seal " tubes. The gradient was fractionated into approximately ten fractions each of 3.3ml.

#### 2.2.2.6. Rate Dependent Sedimentation Gradient Using the M.S.E. 6 x 38ml Swing-Bucket Rotor

Rapid and multiple small-scale rate-dependent banding experiments could be performed using the M.S.E. 6 x 38ml swing-bucket rotor. P.N.S. was prepared as described above for small-scale isopycnic gradients in a minimum volume of 8.5% w/w sucrose. 1ml of P.N.S. was loaded onto a gradient consisting of 5ml volumes of 12, 16, 20, 24 and 28% w/w sucrose resting on a cushion of 7ml 50% w/w and 3ml of 60% w/w sucrose (all containing 3mM imidazole pH 7.2) held in a 38ml thin-walled tube. Centrifugation was at 13,000 r.p.m. ( $g_{\max} = 31,000g$ ) for 11 minutes. After centrifugation the tubes were unloaded by upward displacement with Fluorochemical FC43 using the M.S.E. unloading apparatus. The gradient was fractionated into approximately ten fractions of 3.3ml.

## 2.2.3. Analysis of Fractions

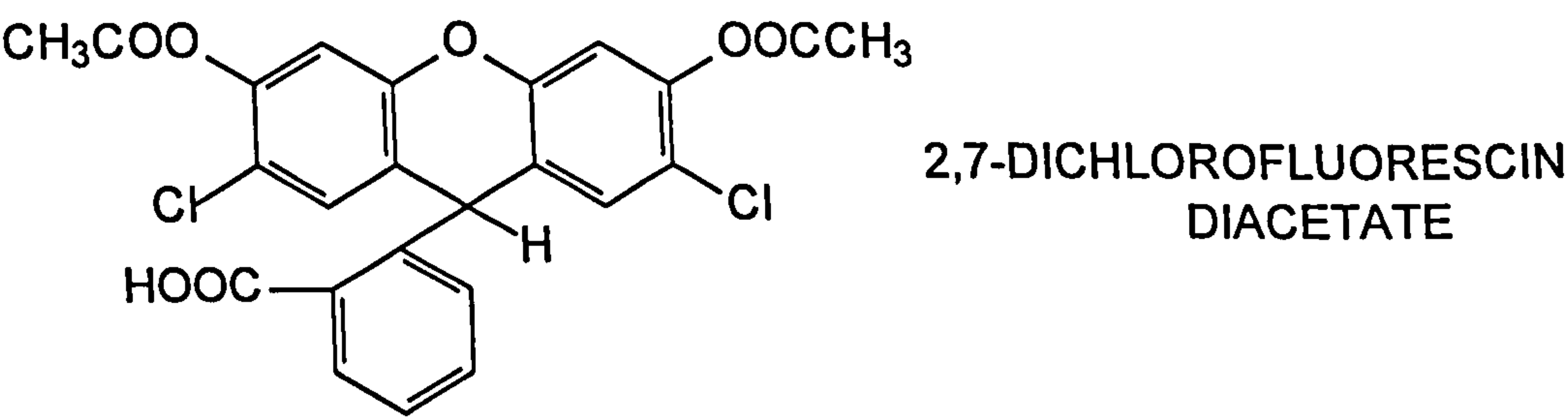
### 2.2.3.1. - Sucrose

Sucrose concentration and density were estimated by the refractive index measurement of the fractions using an Abbé refractometer. R.I. values were converted to concentration and density using tables published in the *Handbook of Chemistry and Physics 56th Edition* (Weast (Ed.) 1975).

### 2.2.3.2. Protein

The protein content of the fractions was estimated by the method of Bradford (1976) using a commercially available dye-concentrate (Biorad cat. no. 500-0006). The assay depends on a shift in absorption maximum of Coomassie Brilliant Blue G250 from 465nm to 595nm when protein is bound by the dye.

0.8ml of a suitable dilution of the subcellular fraction(containing up to 20µg of protein) was mixed with 0.2ml of dye-concentrate and allowed to stand at room temperature for 20 minutes. The absorbance was then read at 595nm against a no-protein blank containing 0.8ml of water and 0.2ml of dye-concentrate. A calibration of 0 - 20µg of standard protein (bovine serum albumin) was prepared each time the assay was performed. This assay had the advantage of being rapid, convenient, sensitive and did not show significant interference caused by the high concentration of carbohydrate in the fractions.



alkaline hydrolysis

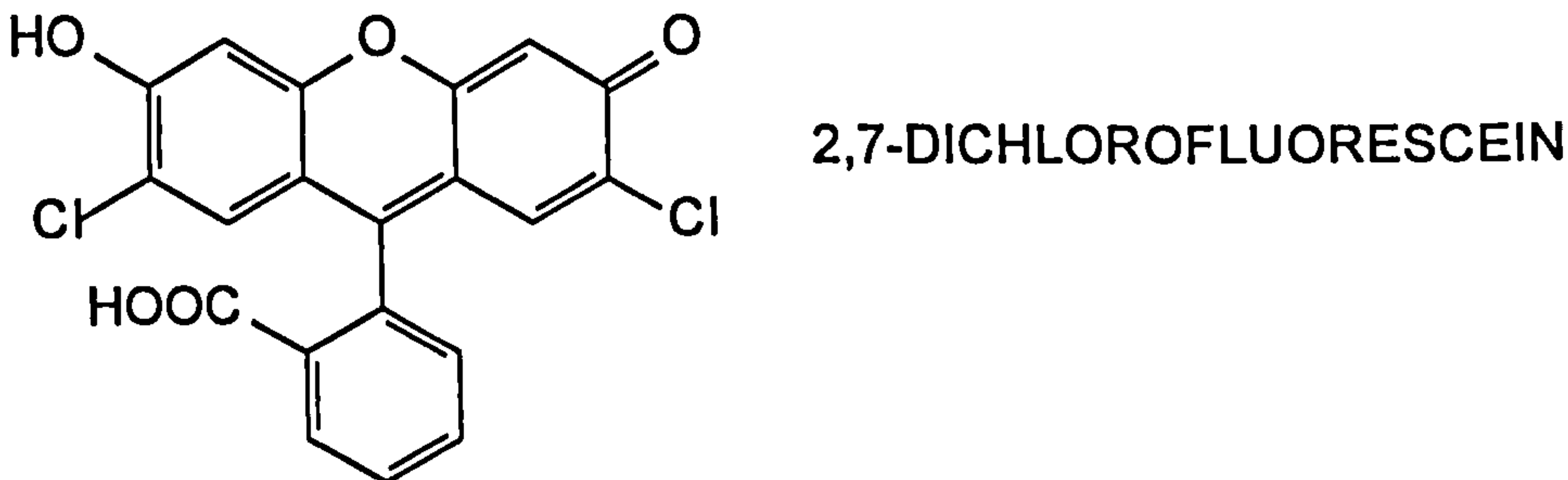
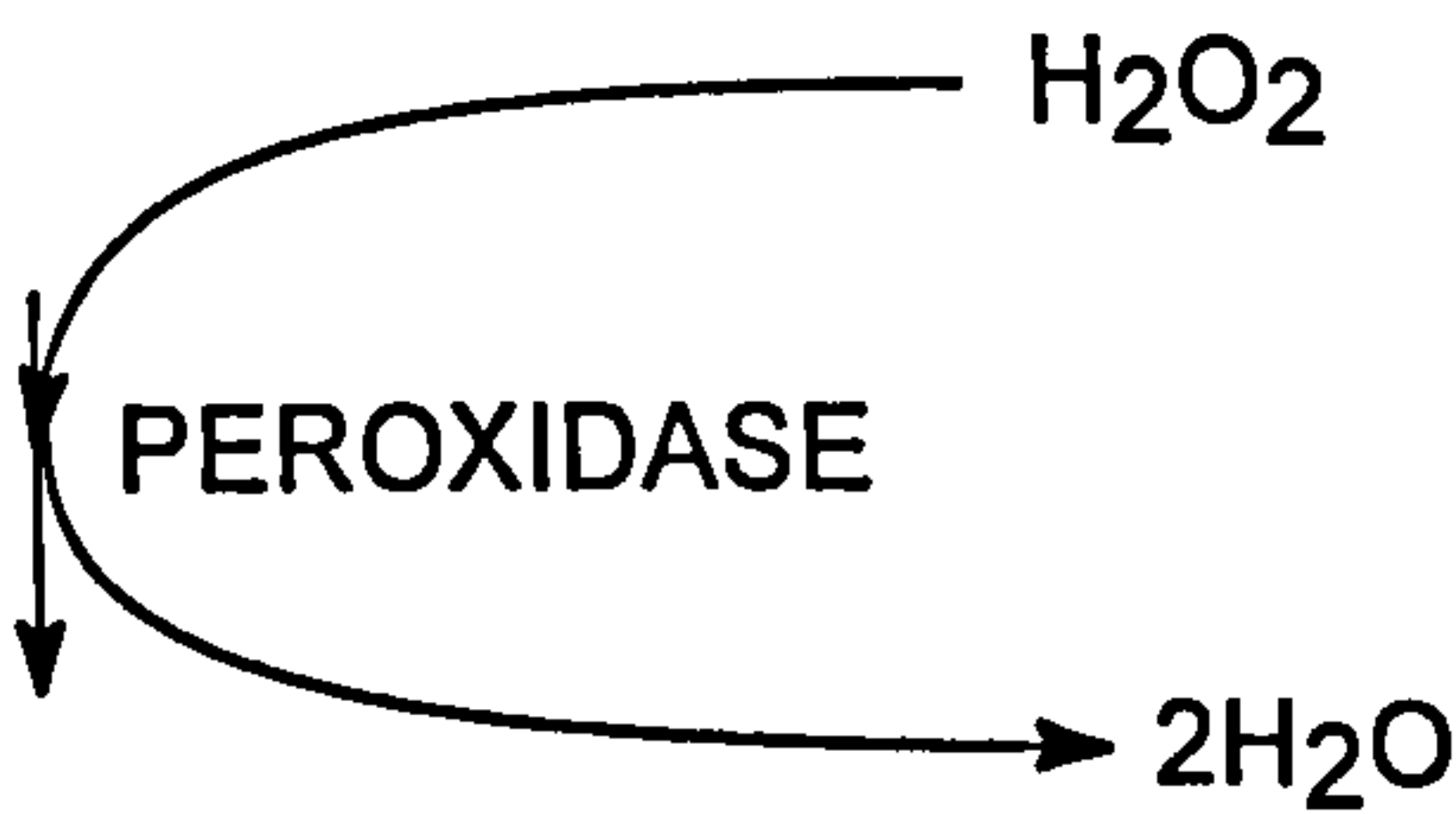
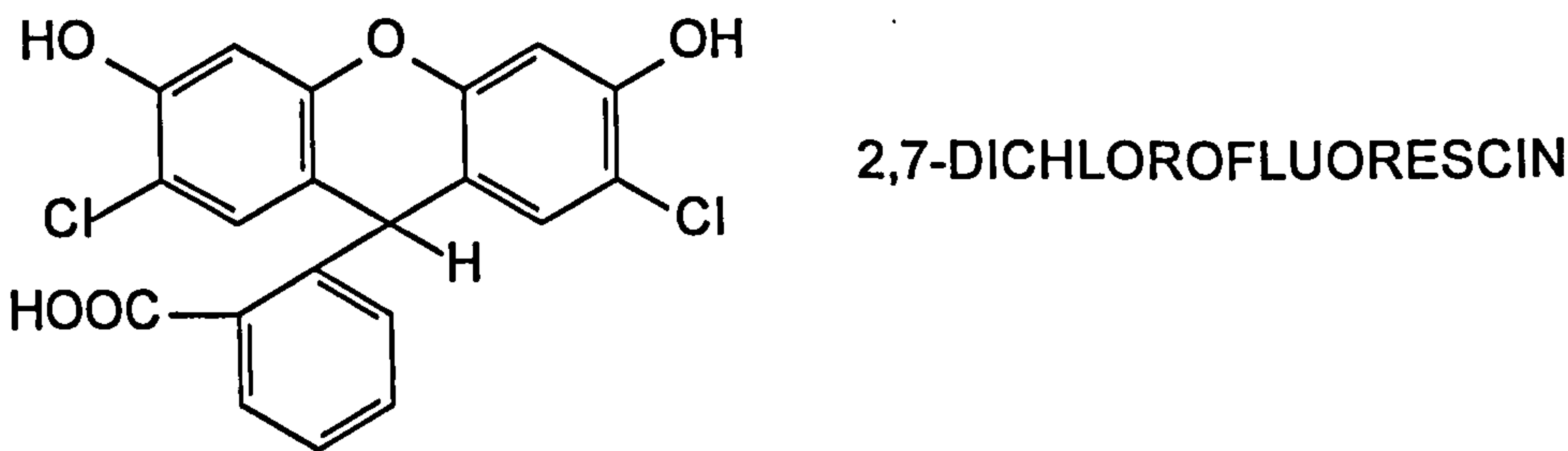


Figure 2.2.3.3.a) - The Peroxidase Linked Oxidation of 2,7 - *l* -Dichorofluorescin  
By Hydrogen Peroxide



### 2.2.3.3. Mannitol Oxidase

#### 2.2.3.3.a) 2,7- *leuco* - Dichlorofluorescin - Peroxidase Linked Assay

Mannitol oxidase activity could be estimated by a slight modification of the spectrophotometric method of Malik *et al* (1987) that measures hydrogen peroxide production. 2,7-*leuco*-Dichlorofluorescin (*l* -DCF) is oxidised by hydrogen peroxide via horseradish peroxidase to 2,7-*l* -dichlorofluorescein (see figure 2.2.3.3.a)) which absorbs strongly at 502nm. The oxidised dye has a high molar extinction coefficient ( $91,000 \text{ M}^{-1} \text{ cm}^{-1}$ ) and a high stoichiometry has been reported (1 mole of hydrogen peroxide produces 5.3 moles of oxidised dye) (Kochli and von Wartburg 1978). Therefore the assay is extremely sensitive with a theoretical molar extinction coefficient of approximately  $480,000 \text{ M}^{-1} \text{ cm}^{-1}$  with respect to hydrogen peroxide.

The cuvette contained 0.16mg of horseradish peroxidase Sigma typeII, 0.05mg of alkali-hydrolysed 2,7-*l* -dichlorofluorescin diacetate, 2mg of 3-amino-1,2,4,-triazole (to inhibit any catalase activity present in the fraction), 10mM potassium phosphate pH 7.4, 40mM mannitol and up to 0.4ml of subcellular fraction in a total volume of 1ml. The cuvette holder was thermostated at 30°C and the reaction followed continuously at 502nm. Appropriate control rates (assays lacking enzyme or substrate) were subtracted from the test rates in the presence of substrate.

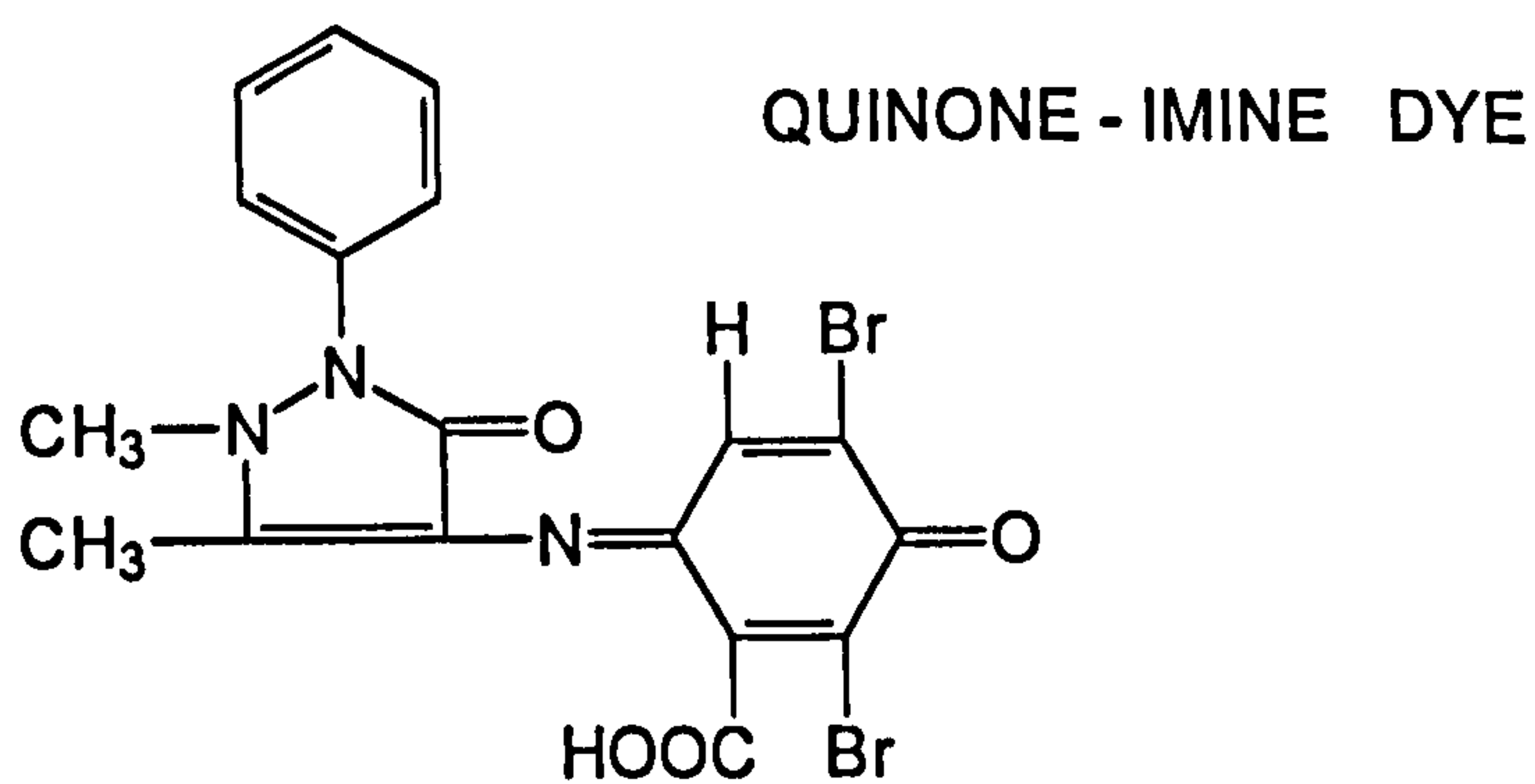
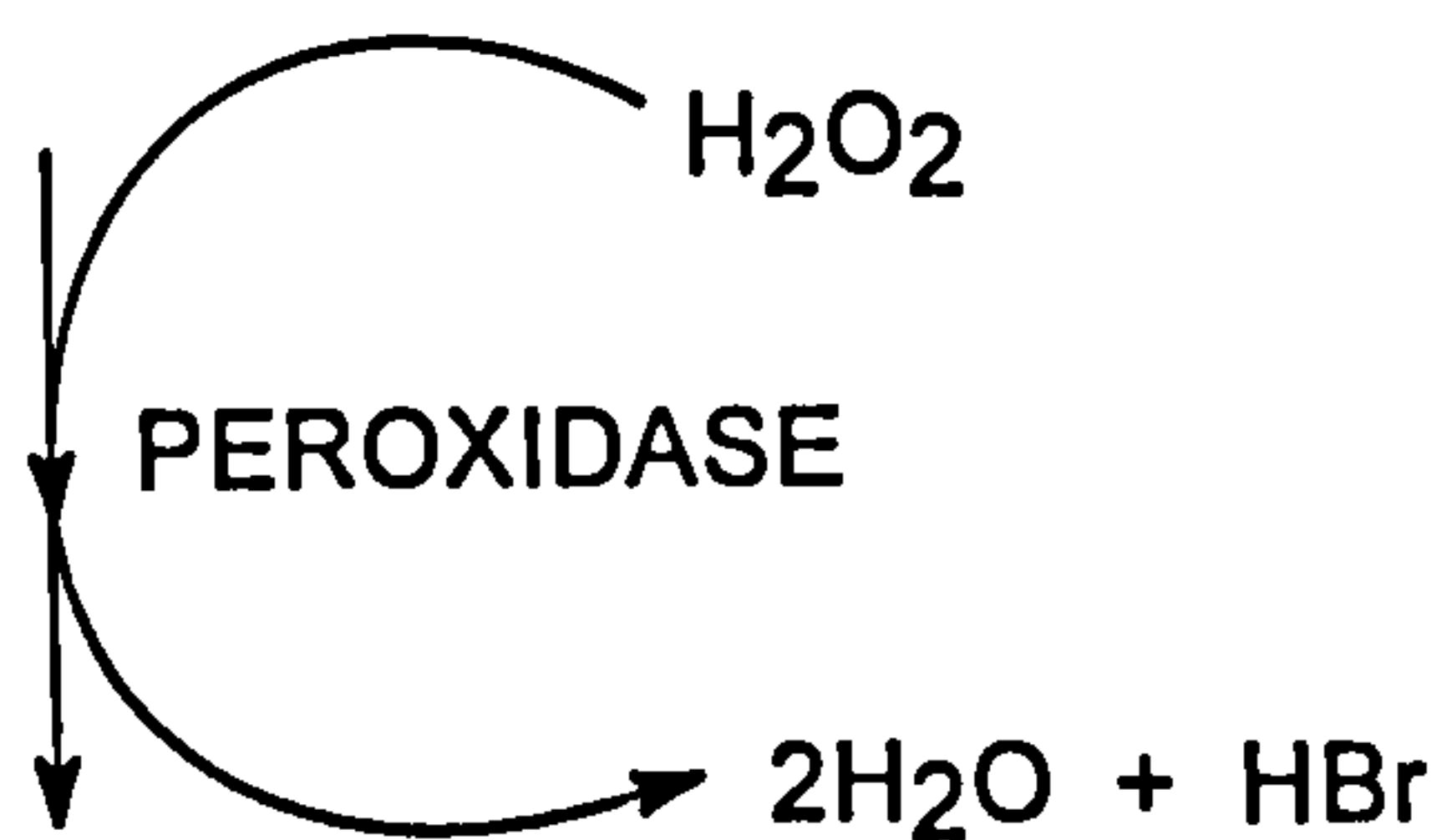
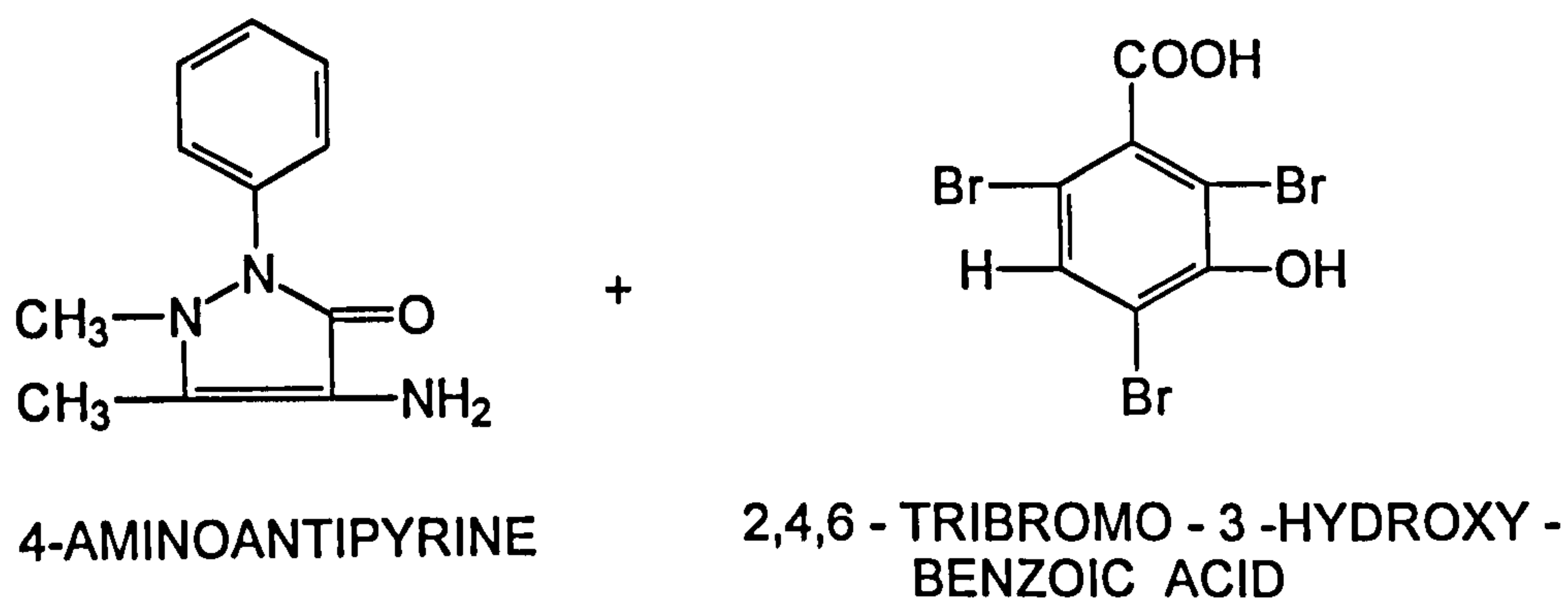


Figure 2.2.3.3.b) - The Mechanism of Dye Formation in the TBHBA-AA Peroxidase Linked Assay

#### 2.2.3.3.b) 2,4,6 -Tribromo-3-hydroxy-benzoic Acid 4-Aminoantipyrine Peroxidase Linked Assay

In later studies mannitol oxidase activity was also estimated by an alternative method. This method also measures hydrogen peroxide production and was based on a method for assaying cholesterol using cholesterol oxidase published by Trinder and Webster (1984). Hydrogen peroxide is utilised via peroxidase for the production of a quinone-imine dye from 4-aminoantipyrine (AA) and a substituted phenol, 2,4,6-tribromo-3-hydroxy-benzoic acid (TBHBA) (figure 2.2.3.3.b)).

A 0.5ml incubation contained up to 0.2ml of subcellular fraction, 0.4mg of TBHBA, 0.1mg of AA, 0.1mg of horse radish peroxidase type II, 20mM potassium phosphate pH 7.4 and 100mM mannitol. Control assays lacking substrate or enzyme were also performed. The tubes were incubated at 23°C (*Helix*, *Limax* and *Arion*) or 30°C (*Achatina*). The reaction was terminated by the addition of 0.5ml of 1% Triton X100 in 1M sodium citrate buffer pH 3.0 (which did not affect the absorbance of the dye product). The absorbance was read at 510nm against the no-enzyme control ( $\epsilon = 29,000 \text{ M}^{-1} \text{ cm}^{-1}$  with respect to hydrogen peroxide). Absorbance was found to be linear with enzyme activity up to two absorbance units.

This method was found to be both reliable and convenient. Although less sensitive when compared to the *l*-DCF-peroxidase linked method relatively low control values were recorded. The linearity (absorbance vs. enzyme activity) was also good and a large number of assays could be performed in a relatively short time.

#### 2.2.3.4. Aromatic Alcohol Oxidase

##### 2.2.3.4.a) 2,7 - *leuco*- Dichlorofluorescein Peroxidase Linked Assay

Aromatic alcohol oxidase activity was estimated by essentially the same method as that previously described for mannitol oxidase using the 1 -DCF-peroxidase linked assay except that 1mM cinnamyl alcohol was substituted for the mannitol. Salicylaldehyde (20mM) was included in both test and control assays to inhibit any aldehyde oxidase activity present from acting on aldehyde present in the substrate (*Arion* and *Limax*). In assays with sample derived from *Helix* and *Achatina* salicylaldehyde was also included at this concentration in both test and control assays to allow for aldehyde oxidase activity that might be present acting on contaminating aldehyde in the substrate. Cuvettes were held in a thermostated holder at 30°C.

##### 2.2.3.4.b) 2,4,6 - Tribromo-3-hydroxy-benzoic acid / 4-Aminoantipyrine -Peroxidase Linked Assay

Aromatic alcohol oxidase could also be more conveniently estimated by essentially the same method as that previously described for mannitol oxidase measuring hydrogen peroxide by linking it via peroxidase to the production of a quinone-imine dye from 4-aminoantipyrine and a substituted phenol, 2,4,6,-tribromo-3-hydroxy-benzoic acid. Cinnamyl alcohol (1mM) (*Arion*, *Helix* and *Limax*) or crotyl alcohol (1mM) (*Achatina*) were substituted for mannitol. Salicylaldehyde (40µM) was included in both test and control assays for the reasons mentioned above. Assays were incubated at 23°C (*Arion*, *Helix* and *Limax*) or 30°C (*Achatina*).



#### 2.2.3.5. Semicarbazide-Sensitive Amine Oxidase

Semicarbazide-sensitive amine oxidase activity was estimated by the TBHBA-AA-peroxidase linked method previously described for mannitol oxidase except that 80 $\mu$ M benzylamine was substituted for mannitol. Subcellular fractions were assayed in the presence (control) or absence (test) of 100mM semicarbazide. No semicarbazide-insensitive activity was detectable. Appropriate blanks lacking enzyme, substrate or semicarbazide were included. The assays were incubated at 30°C.

#### 2.2.3.6. Succinate Dehydrogenase

Succinate dehydrogenase is a flavoprotein localised on the inner-mitochondrial membrane and catalyses the oxidation of succinate to fumarate. Succinate dehydrogenase activity (expressed as succinate-INT reductase) was assayed using the artificial electron acceptor 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) which yields a water-insoluble formazan dye when reduced. The method of Pennington (1961) as modified by Prospero (1974) was used. Up to 100 $\mu$ l of subcellular fraction was mixed with an equal volume of reaction mixture which contained 100mM potassium phosphate pH 7.4, 0.5mM E.D.T.A., 5mg ml<sup>-1</sup> bovine serum albumin, 3.3mM INT and either 80mM sodium succinate (test assays) or 80mM sodium malonate (control assays). The tubes were incubated at 37°C. The reaction was terminated by the addition of 3ml of "stop mix" (a mixture of 20 parts ethyl acetate, 13 parts ethanol and 2 parts 10% w/v aqueous trichloroacetic acid). The tubes were centrifuged (3,000 r.p.m. in a bench centrifuge for 10 minutes) to remove denatured protein and the absorbance of the single-phase supernatant read at 490nm. The succinate dehydrogenase activity was proportional to the absorbance of the test assay minus that of the control assay.

### 2.2.3.7. Catalase

Catalase activity was estimated by a slight modification of the method of Baudhuin *et al* (1964) that measures the catalatic breakdown of hydrogen peroxide. The measurement of hydrogen peroxide concentration is dependent on the formation of a coloured complex (peroxy titanium sulphate) with acidified titanium sulphate (Patel and Mohan 1960). A solution of buffered hydrogen peroxide was prepared by adding sufficient 20vols. hydrogen peroxide to 20mM imidazole buffer pH 7.2 (ice-cold) such that when 3ml of this solution was added to 2ml of 5% titanium sulphate (in 0.25M sulphuric acid) the absorbance at 410nm (read against distilled water) was approximately 1.80. This was equivalent to a hydrogen peroxide concentration of 2.8mM. Up to 0.2ml of subcellular fraction was treated with 0.2ml of 2% Triton X100. To this was added 3ml of ice-cold buffered substrate (prepared as described above) followed by incubation on ice (0°C) for 10 minutes. A no-enzyme control (in triplicate) was included to determine the "zero-consumption" of substrate. The reaction was terminated by the addition of 2ml of 5% titanium sulphate (in 0.25M sulphuric acid) (filtered before use). The tubes were allowed to equilibrate to room temperature, centrifuged at 3,000 r.p.m. (in a bench centrifuge) for 20 minutes and the absorbance read at 410nm against distilled water. Absorbances of approximately 1.8 for the "zero-consumption" and not less than 0.2 for the test assays were required to ensure a reliable estimation of catalase activity.

As the breakdown of hydrogen peroxide follows first-order kinetics one unit of enzyme activity is defined as the amount of enzyme causing the destruction of 90% of the substrate in 1 minute. Units of catalase activity were calculated by the formula :-

$$\text{units of activity} = \log_{10} (1000 A_{zc}) - \log_{10} (1000 A_t)$$

where  $A_{zc}$  is the absorbance of the "zero-consumption" and  $A_t$  is the absorbance of the test assay at 410nm.

#### 2.2.3.8. Alkaline Phosphatase

Alkaline phosphatase activity was estimated by a modification of the method of Babson *et al* (1966) but using 4-nitrophenyl phosphate as substrate (Bessey *et al* 1946). A 1ml incubation contained up to 0.2ml of subcellular fraction, 25mM sodium fluoride (to inhibit acid phosphatase), 0.04% Triton X100, 5mM magnesium chloride, 0.2M 2-amino-2-methyl-1-propanol hydrochloride pH 10.2 and 10mM 4-nitrophenol phosphate. A no-enzyme control assay was also included. After incubation at 37°C the reaction was terminated by the addition of 1ml of 1M sodium hydroxide. The absorbance was read at 405nm (against the no-enzyme control) which was proportional to the 4-nitrophenol released.

#### 2.2.3.9. Aryl Sulphatase

Aryl sulphatase activity was estimated by a slight modification of the method of Baum *et al* (1959). A 0.4ml incubation contained up to 0.2ml of subcellular fraction, 0.1% Triton X100, 0.25M sodium acetate buffer pH 5.2, 1mM sodium chloride, 0.6mM sodium pyrophosphate and 5mM *p*-nitrocatechol sulphate (2-hydroxy-5-nitro-phenyl sulphate). A no-enzyme control assay was also included. After incubation at 37°C the reaction was terminated by the addition of 0.6ml of 1M sodium hydroxide which also allowed the visualisation of the *p*-nitrocatechol released by the reaction. The absorbance was read at 515nm against the no-enzyme control. The absorbance was found to be linear with enzyme activity up to an absorbance of at least 0.4. The molar extinction of *p*-nitrocatechol at alkaline pH is 12,400 M<sup>-1</sup>cm<sup>-1</sup>.

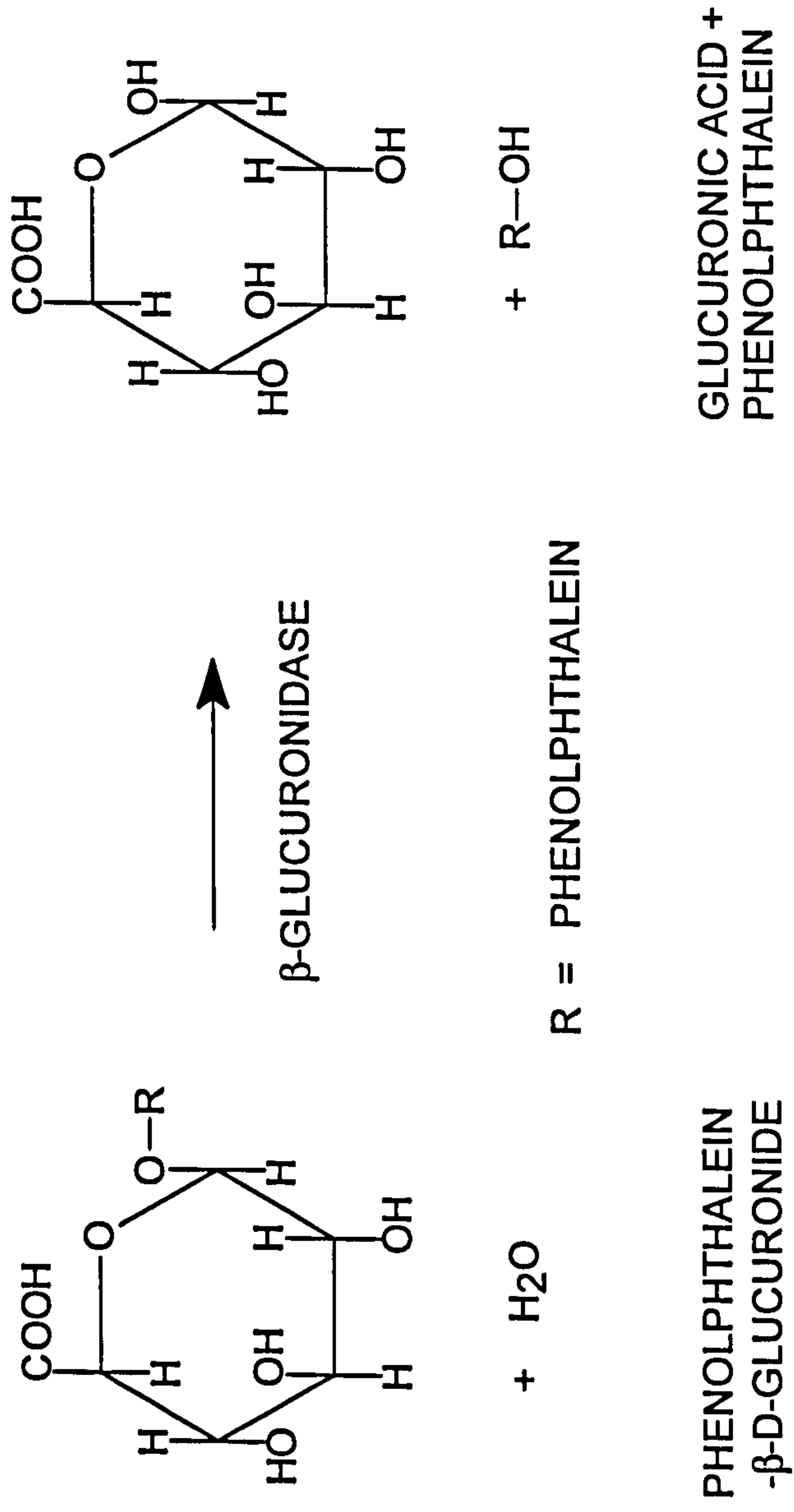


Figure 2.2.3.10. - The Hydrolysis of Phenolphthalein-β-D-glucuronide by β-Glucuronidase



### 2.2.3.10. $\beta$ -Glucuronidase

$\beta$ -D-Glucuronidase activity was estimated by a slight modification of the method of Stahl and Fishman (1984). The method utilises phenolphthalein- $\beta$ -D-glucuronide as the substrate which is hydrolysed by the enzyme to produce glucuronic acid and phenolphthalein. The latter product is coloured at high pH and can be measured spectrophotometrically (figure 2.2.3.10.). The assay medium contained 0.2M sodium acetate buffer pH 4.5, 0.1% Triton X100 and up to 0.2ml of subcellular fraction. The reaction was initiated by the addition of 0.1ml of 10mM substrate to give a final assay volume of 0.5ml. A no-enzyme control assay was also included. The assays were incubated at 37°C. The reaction was terminated by the addition 1.0ml of 0.2M glycine-NaOH buffer pH 11.7 containing 0.2% S.D.S. . This was followed by the addition of 1.5ml of water. The absorbance was read at 540nm and was proportional to the phenolphthalein released. This method was found to be linear with enzyme activity up to an absorbance of at least 0.4. A calibration of 0 -30 nmols phenolphthalein (in 3ml) was also made. An absorbance of 0.1 was found to equivalent to 3.1 $\mu$ g of phenolphthalein released per assay volume.

### 2.2.3.11. Esterase

Esterase activity was estimated by a modification of the method of Ravin and Seligman (1953) for measuring pancreatic lipase activity. 1-Naphthyl acetate was utilised as the substrate for esterase. Buffered substrate was prepared by dissolving 10mg of 1-naphthyl acetate in 10ml of acetone which was then added with stirring to 100ml of 50mM veronal buffer pH8.0 to give a substrate concentration of 0.5mM. Up to 0.1ml of subcellular fraction was added to 2.0ml of buffered substrate and incubated at 20°C.

1-Naphthol released by the reaction was visualised by the addition of a diazo salt, Fast Red ITR. A solution of 10mg ml<sup>-1</sup> Fast Red ITR was prepared in 1 volume of dimethylformamide and 4

volumes of 0.4M sodium acetate buffer pH 5.0. At the end of the incubation time 0.4ml of buffered Fast Red ITR (10mg ml<sup>-1</sup>) was added to the assay tube and left for a further 5 minutes to allow the azo-dye to develop. 3ml of "stop-mix" (as described for succinate dehydrogenase section 2.2.3.6.) was then added and the tube centrifuged at 4,000 r.p.m. for 20 minutes in a bench centrifuge to remove denatured protein. The absorbance of the single-phase supernatant was read at 540nm against a no-enzyme blank. Absorbance was proportional to enzyme activity up to an absorbance of at least 0.4.

### 2.2.3.12. Acid Phosphatase

Acid phosphatase activity was estimated by a method based on that of Hubscher and West (1965) which measures the fluoride-sensitive hydrolysis of 1-naphthyl phosphate. An assay volume of 1ml contained 0.1% Triton X100, 0.04M sodium acetate buffer pH 5.0, 2mM E.D.T.A. (to inhibit alkaline phosphatase), 10mM 1-naphthyl phosphate and up to 0.4ml of subcellular fraction. Control assays were also performed and these additionally contained 20mM sodium fluoride. Incubation was at 37°C. 1-Naphthyl phosphate released was visualised by essentially the same method as that described for esterase (see above). At the end of the incubation 0.64ml of 10mg ml<sup>-1</sup> Fast red ITR in 20% v/v aqueous dimethylformamide was added to the assay and the colour allowed to develop for exactly 5 minutes. 4.8ml of "stop-mix (as described for succinate dehydrogenase section 2.2.3.6.) was added to the tube which was then centrifuged at 4,000 r.p.m. for 20 minutes in a bench centrifuge to remove denatured protein. The absorbance of the single-phase supernatant was read at 540nm. The absorbance is proportional to enzyme activity up to an absorbance of at least 0.4. The acid phosphatase activity is defined as the fluoride-sensitive activity and is calculated by subtracting the control value (assayed in the presence of 20mM sodium fluoride) from the test value (assayed in the absence of fluoride).

### 2.2.3.13. NADPH - Cytochrome c Reductase

NADPH-cytochrome c reductase activity was estimated by following the NADPH-dependent reduction of cytochrome c spectrophotometrically at 550nm. 225 $\mu$ l of suitably diluted subcellular fraction was added to 450 $\mu$ l of medium at 30°C containing 40mM sodium phosphate buffer pH 7.2, 40mM potassium chloride, 680mM sodium chloride, 64mM nicotinamide, 0.5mM potassium cyanide, 0.12% Triton X100 and 0.1mM of oxidised cytochrome c (Beaufay *et al* 1974a). The change in absorbance was monitored continuously at 550nm in a thermostated cuvette holder at 30°C. This corresponded to the NADPH-independent reduction of cytochrome c (ie - the control rate). After approximately 5 minutes 75 $\mu$ l of 3mM NADPH (to give a final concentration of 0.3mM in the cuvette) was added and the change in absorbance again monitored continuously. This was the test rate. The NADPH-dependent cytochrome c reductase activity was obtained by subtracting the control rate from the test rate.

### 2.2.3.14. Thiamine Pyrophosphatase

Thiamine pyrophosphatase activity was estimated by a modification of the method of Morré (1971). Inorganic phosphate released by the hydrolysis of thiamine pyrophosphate was estimated according to the method of Lanzetta *et al* (1979). The assay tube contained 33mM sodium barbitone buffer pH 7.8, 15mM calcium chloride, 5mM E.D.T.A. (to inhibit alkaline phosphatase), 13mM potassium fluoride (to inhibit acid phosphatase), 0.1% Triton X100, 3mM thiamine pyrophosphate and up to 0.3ml of subcellular fraction in a total volume of 1ml. Suitable control assays were included. After a suitable incubation time (usually overnight) a 50 $\mu$ l aliquot was removed from the assay and this was used to determine the inorganic phosphate present.

A 50 $\mu$ l sample (either test or control) was added to 0.8ml of a colour reagent containing 1% w/v ammonium molybdate, 0.03% w/v malachite green, 0.08% w/v synperonic (a detergent) and 1M hydrochloric acid. This reagent halted the reaction. After exactly 2 minutes 0.1ml of 34% w/v sodium citrate was added and the tubes allowed to stand at room temperature for 1 hour. After this time they were diluted with 1.05ml of distilled water. The absorbance was then read at 662nm. Thiamine pyrophosphatase activity was calculated by subtracting the appropriate control values from the test value. A calibration of 0 - 25 nmoles of inorganic phosphate was constructed on each occasion that the assay was performed.

Due to the sensitivity of the inorganic phosphate assay new disposable plastic tubes and cuvettes were used throughout. Care was also taken to use only acid washed glassware, high grade chemicals and good quality distilled water in the preparation of reagents for this assay.



## 2.2.4. Presentation of Fractionation Results

The distribution of the enzyme activities in the fractions is given in the form of frequency histograms according to De Duve (1967). The ordinate ("frequency") represents the relative concentration of the enzyme in the fraction. The relative concentration is defined as the concentration of the enzyme in the fraction divided by the concentration if it were homogeneously distributed throughout the whole volume unloaded. The abscissa represents the cumulative volume of the fractions (at increasing density) as a percentage of the total volume unloaded. In experiments where the first fraction volume ("sample layer") was very large compared to that of the gradient this fractions volume has been arbitrarily represented as 25% of the total volume.

## 2.2.5. Electron Microscopy

Samples of subcellular fractions were fixed overnight in 5% glutaraldehyde, 100mM cacodylate buffer pH7.2 which also contained sucrose at the same concentration as that of the isolated fraction. After fixation the samples were diluted with a slightly lower concentration of sucrose and pelleted by centrifugation. The pellets were washed over a period of two days with sucrose solutions (in 100mM cacodylate buffer pH 7.2) of gradually decreasing concentration and finally with 100mM cacodylate buffer pH 7.2. This was to avoid subjecting the samples to osmotic shock to maintain the morphology of the isolated organelles. Pellets were post-fixed with 1% osmium tetroxide and embedded in Taab resin. Sections were stained with uranyl acetate and lead citrate.

## 2.2.6. S.D.S. - P.A.G.E. Analysis

S.D.S.-P.A.G.E. analysis was performed according to the method of Laemmli (1970). Samples of the subcellular fractions were centrifuged at 100,000g for one hour to pellet the organelles. The pellets were suspended in a sample buffer containing 0.2M tris-HCl pH 6.8, 8mM E.D.T.A., 0.5% v/v 2-mercaptoethanol, 0.0025% w/v bromophenol blue, 8% w/v S.D.S. and 25% w/v sucrose. This was heated to 90°C for 10 minutes to denature the proteins fully.

An appropriate volume (2 -15ml) was loaded onto a 12% pre-cast gel (Biorad "mini-protean II" 12% gel cat.no. 161-0901). Electrophoresis was at 200 volts until the bromophenol blue had migrated to the bottom of the gel (approximately 1 hour). Gels were stained in an aqueous fixing solution of 7.5% v/v acetic acid, 50% v/v methanol containing 0.25 w/v Coomassie Brilliant Blue. Gels were destained overnight in 7% v/v acetic acid, 10% v/v methanol in water.

## 2.3. Sample Preparation And Characterisation of Catalytic Activities

### 2.3.1. Preparation of Samples for Enzyme Analysis

#### 2.3.1.1. Aromatic Alcohol Oxidase

Aromatic alcohol oxidase in molluscs is a membrane-bound activity that can be detected in crude tissue homogenates. Both particulate and solubilised enzyme samples were used for experiments and these were prepared as described below.

##### 2.3.1.1.a) Crude Membrane Preparation

Digestive gland was dissected from several animals and homogenised in 10% w/w sucrose containing 3mM imidazole pH7.2. This was subjected to low speed centrifugation at 3,000 r.p.m. for 10 minutes to remove cell debris and connective tissue. The supernatant was then centrifuged at 100,000g for 2 hours to quantitatively sediment the membranes. The pellet was resuspended in a small volume of 10% w/w sucrose (containing 3mM imidazole pH 7.2) and gently homogenised to ensure even dispersal of the membrane fragments. The membrane fraction provided a useful source of activity.

### 2.3.1.1.b) Detergent Solubilisation Experiments

Experiments were conducted to test the efficacy of a range of detergents in solubilising aromatic alcohol oxidase activity. A small volume of crude membrane preparation (prepared as described above) containing approximately 6mg of protein was dispersed in 5ml of varying concentrations of a detergent. A zero-detergent control experiment was also conducted. This was centrifuged at 100,000g for 2 hours. The supernatant was decanted and the pellet resuspended in 1ml of 10% w/w sucrose. The activity was then estimated in the pellet and the supernatant. The values were compared to estimate the percentage solubilisation achieved by the detergent at that concentration. The percentage inactivation brought about by the detergent (if any) was estimated by comparing the combined activity in the pellet and supernatant with the activity in the no-detergent control (the activity remaining in the pellet for the no-detergent control was defined as 100%). The following detergents were tested :-

Nonionic - Triton X100

Brij 35

Lubrol WX

Nonidet P40

Tween 20

*n*-Octyl- $\beta$ -D-glucoside

Cationic - Cetyl-trimethyl-ammonium bromide

Anionic - Sodium deoxycholate

Sodium cholate

Zwitterionic - CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-  
1-propane sulphonate)



### 2.3.1.1.c) Preparation of Solubilised Aromatic Alcohol Oxidase Activity

Arising from the experiments described above a method was devised for producing a soluble aromatic alcohol oxidase preparation in good yield and enriched at least 20-fold with respect to the digestive gland homogenate. Crude membrane preparation prepared as described above was dispersed in a large volume of 0.5% Tween 20, 10mM potassium phosphate pH 7.4 and centrifuged at 100,000g for 2 hours. The supernatant, which contained less than 3% of the total activity, was discarded. The pellet was resuspended in 10mM potassium phosphate pH 7.4, centrifuged at 100,000g for 2 hours and the supernatant discarded. This time the pellet was resuspended in a small volume of between 0.5% and 1.0% w/v Nonidet P40 and again centrifuged at 100,000g for 2 hours. The supernatant contained typically 80% (*Arion*, *Helix* and *Limax*) or >98% (*Achatina*) of the activity therefore the pellet was discarded. This solubilised and enriched enzyme preparation was completely free of contaminating aldehyde oxidase activity and was used for most experiments.

### 2.3.1.1.d) DEAE- Sepharose Filtration of Solubilised Aromatic Alcohol Oxidase

The solubilised activity obtained by the method above could be filtered through a small column containing DEAE-sepharose equilibrated with 0.1% Nonidet P40, 25mM potassium phosphate. This gave a further 3-fold enrichment of the aromatic alcohol oxidase activity giving a total purification of 60-fold with respect to the homogenate. However the yield was relatively poor (approximately one third of the activity was typically lost) so this step was usually omitted

### 2.3.1.2. Aldehyde Oxidase

Aldehyde oxidase is a soluble cytosolic enzyme. Activity could be detected in tissue homogenates. However for most experiments activity was obtained from the soluble fraction of tissue homogenates. Digestive gland, kidney (*Achatina* only) or liver (from albino mouse, used for comparison) was dissected from animals and homogenised in 10% w/w sucrose containing 3mM imidazole pH7.2. The homogenate was centrifuged at 100,000g for 2 hours. All of the activity was found in the supernatant. The soluble fraction was used for most experiments without further purification.

### 2.3.1.3. Mannitol Oxidase

Mannitol oxidase activity is membrane-bound and can be detected in tissue homogenates. However for most experiments the enzyme source was derived from the peak mannitol oxidase fractions after isopycnic centrifugation using digestive gland P.N.S.. For *Arion ater*, *Limax flavus* and *Helix aspersa* a small scale gradient in a Beckman Ti-50 rotor was employed as previously described (see section 2.2.2.5). For *Achatina fulica* a large scale gradient in a BXIV rotor with a B29 insert was used (as described in section 2.2.2.2.). This particulate activity was found to be suitable to use in experiments therefore detergents were not employed to solubilise the activity. The peak mannitol oxidase fractions were approximately 13-fold enriched with respect to the P.N.S. using the small-scale method (*Arion*, *Helix* and *Limax*) and 9-fold using the large-scale method (*Achatina fulica* only).

## 2.3.2. Assay Methods

### 2.3.2.1. Aromatic Alcohol Oxidase

Aromatic alcohol oxidase activity could be estimated by the measurement of hydrogen peroxide production, aldehyde production and oxygen consumption. Several reliable methods were developed to estimate this activity.

#### 2.3.2.1.a) 2,7 -*l* -Dichlorofluorescein Peroxidase Linked Assay

Aromatic alcohol oxidase activity could be routinely assayed by monitoring hydrogen peroxide production using the *l* -DCF-peroxidase linked method previously described (section 2.2.3.4.a)). All reagents were at the concentrations previously described except that salicylaldehyde was omitted when solubilised aromatic alcohol oxidase was used as the enzyme source since this preparation was aldehyde oxidase free.

Several other modifications were also made where appropriate. For the initial substrate comparisons with activity derived from *Arion ater* substrates were added as a solution in methanol to give a final substrate concentration of 1.7mM; the assay medium also contained methanol such that the final concentration of methanol in the assay was 15% v/v. This was to improve the solubility of potential substrates that were only slightly soluble in aqueous media. Methanol at 15% v/v had no detectable effect on the activity of the enzyme. For the comparative experiments using activity derived from several species the potential substrates were supplied in totally aqueous phase at 1mM. Kinetic studies were also in totally aqueous phase. Control assay lacking substrate or enzyme were always included. The assays were performed in a thermostated cuvette-holder at 25°C (*Arion*, *Limax* and *Helix*) or 30°C (*Achatina*).

#### 2.3.2.1.b) 2,4,6 -Tribromo-3-hydroxy-benzoic acid 4-Aminoantipyrine Peroxidase Linked Assay - End-point Method

Aromatic alcohol oxidase activity could be more rapidly estimated by the TBHBA-AA peroxidase linked assay previously described (section 2.2.3.4.b)). The reagents were at the concentration previously stated except that salicylaldehyde was omitted when solubilised activity was used as the enzyme source. For substrate comparison experiments (*Achatina fulica* only) substrates were supplied initially in totally aqueous phase at 100mM (C<sub>1</sub> -C<sub>5</sub> aliphatic alcohol) or 10mM (crotyl and aromatic alcohols). Fatty alcohols (C<sub>6</sub> -C<sub>16</sub>) were dissolved in methanol (5mM stock solution) and 25µl of this stock solution added to the 0.5ml incubation (which also contained 25µl of methanol to aid the solubility of the alcohols) giving a final concentration of 250µM substrate and 10% v/v methanol. Activity with the fatty alcohols was compared to activity with 250µM amyl alcohol and cinnamyl alcohol supplied in the same way; an additional control assay containing 50µl of methanol was also included. Assays were incubated at 30°C and the reaction stopped by the addition of 0.5ml of 1% Triton X100 in 1M sodium citrate buffer pH 3.0.

A modification of the above method was made to enable activity with dodecanol and decanol (which lack significant solubility in aqueous media) in totally aqueous phase. In these assays reagents were at the standard concentrations but in a total volume of 5ml. 50µl of liquid substrate was added to the tubes which were shaken continuously at 30°C to disperse the substrate. The reaction was terminated by the addition of an equal volume of 10% w/v S.D.S. and centrifuged at 3,000 r.p.m. for 20 minutes in a cooled rotor (4°C) to pellet denatured protein. The unused substrate solidified at the temperature and floated to the surface during centrifugation. The clear supernatant was used for absorbance measurement.



Assays where activity was to be estimated in crude tissue homogenates were essentially by the standard method using 1mM cinnamyl alcohol as the substrate except that they also included 0.2% w/v 3-amino-1,2,4-triazole to inhibit catalase activity present. Test and control (lacking alcohol substrate) assays contained 200mM benzaldehyde to overcome the effect of aldehyde oxidase activity acting on contaminating cinnamaldehyde in the substrate. Alcohol oxidase activity was calculated as follows :-

Test result = aldehyde oxidase + alcohol oxidase activities

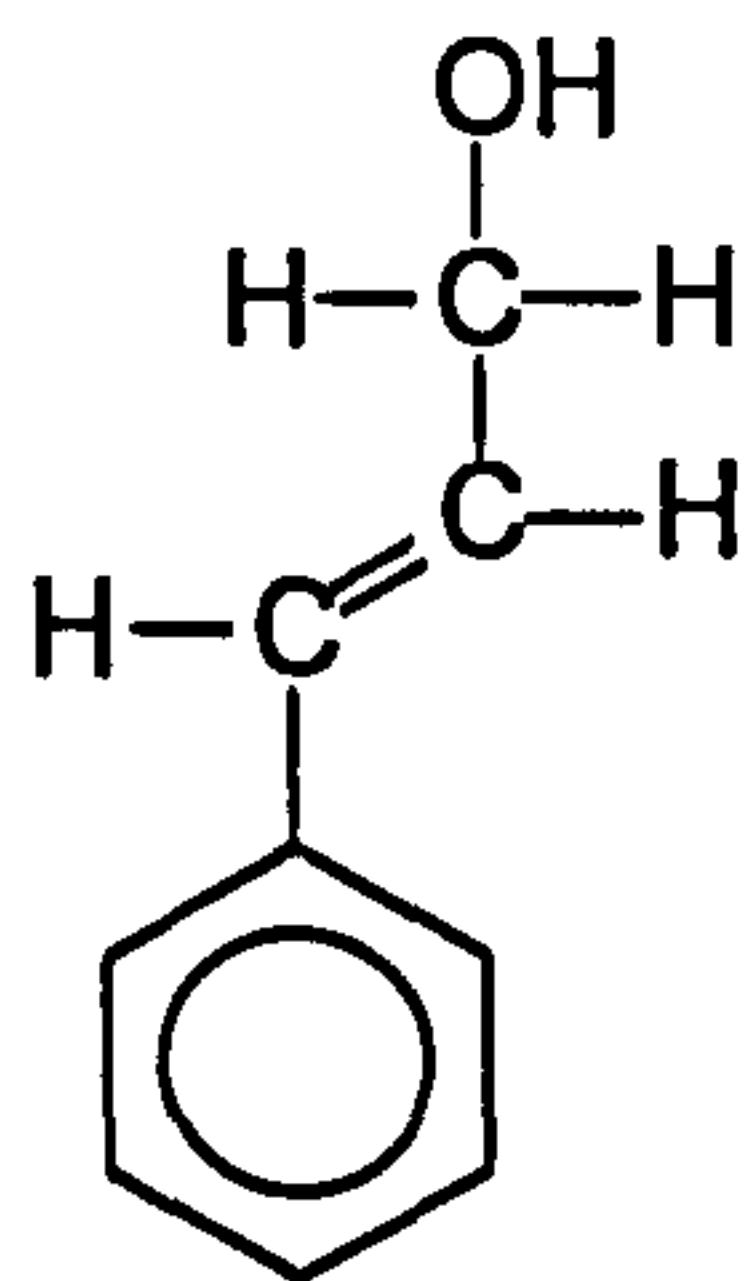
Control result = aldehyde oxidase activity

Test - Control = alcohol oxidase activity

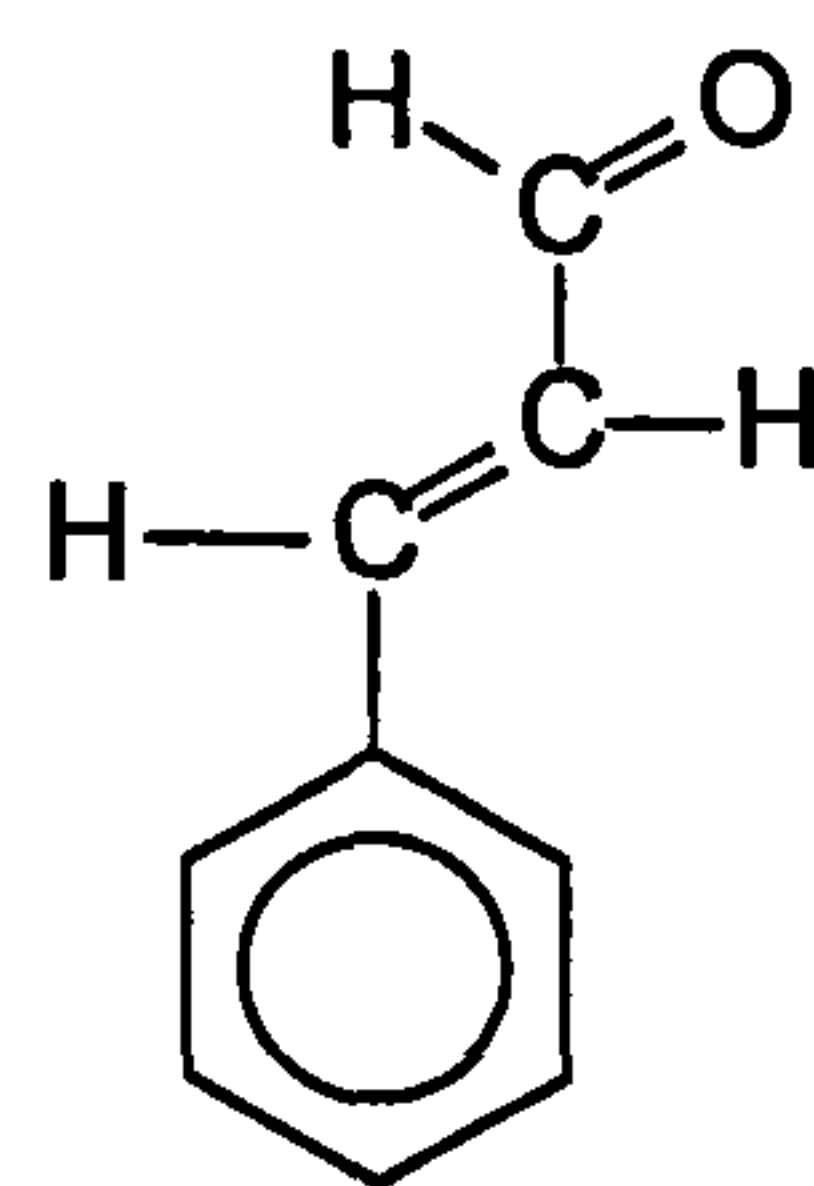
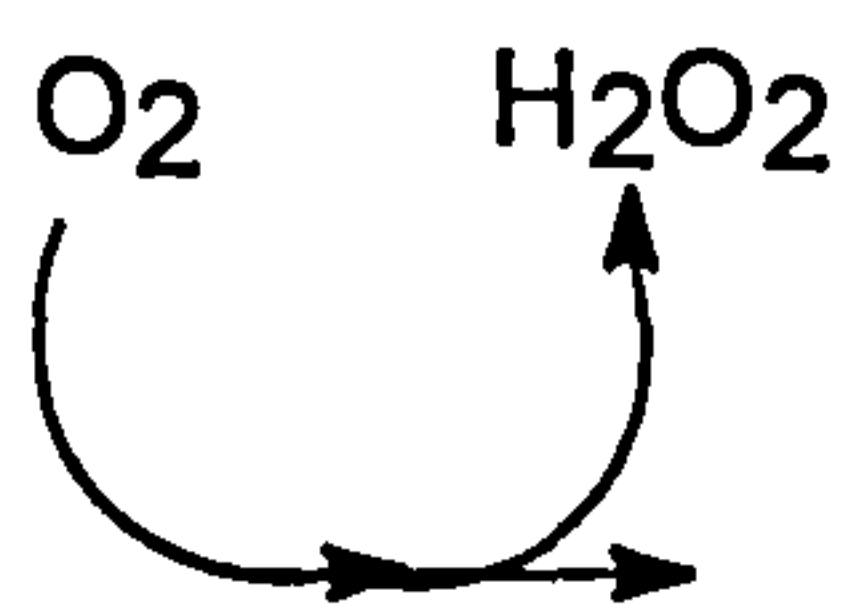
In all experiments the absorbance was read at 510nm ( $\epsilon = 29,000 \text{ M}^{-1} \text{ cm}^{-1}$  with respect to hydrogen peroxide).

#### 2.3.2.1.c) 2,4,6 - Tribromo-3-hydroxy-benzoic Acid 4-Aminoantipyrine Peroxidase Linked Assay - Continuous Method

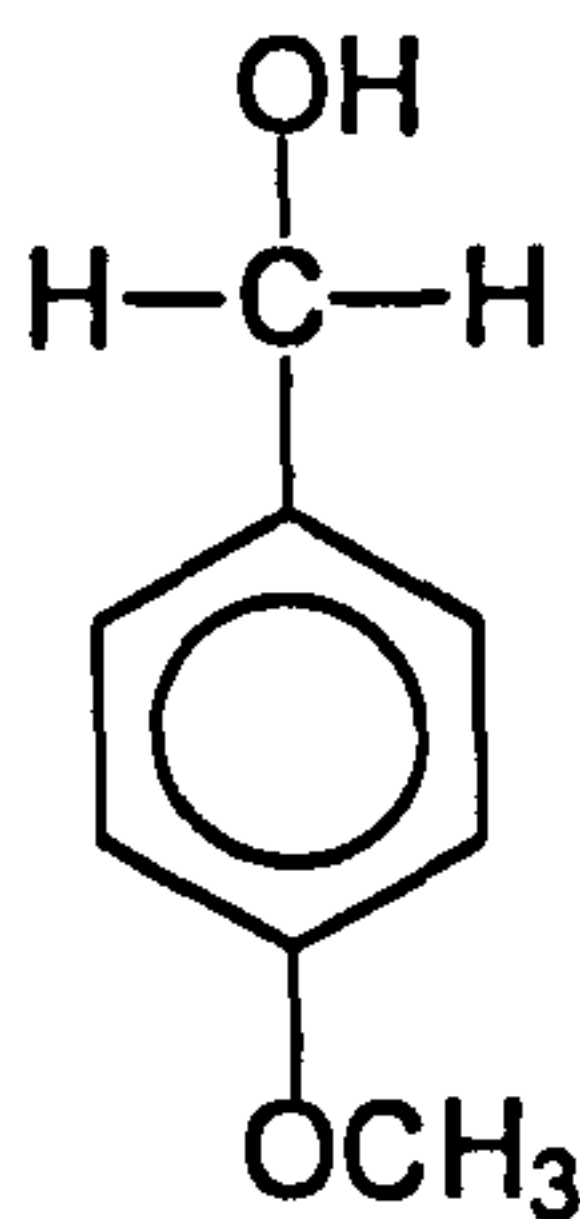
The assay described in the previous section could be adapted for the continuous measurement of hydrogen peroxide production. This method was used for stoichiometry estimations where hydrogen peroxide production was compared to aldehyde production. The cuvette contained 1.0mg ml<sup>-1</sup> TBHBA, 0.2 mg ml<sup>-1</sup> 4-aminoantipyrine, 0.2 mg ml<sup>-1</sup> horse radish peroxidase type II, 33mM potassium phosphate buffer pH 7.4 and 0.333mM cinnamyl alcohol in a total volume of 3ml. The production of hydrogen peroxide was followed continuously at 510nm in a thermostated cuvette-holder at 25°C ( $\epsilon = 29,000 \text{ M}^{-1} \text{ cm}^{-1}$ ). Suitable control assays lacking substrate or enzyme were also performed.



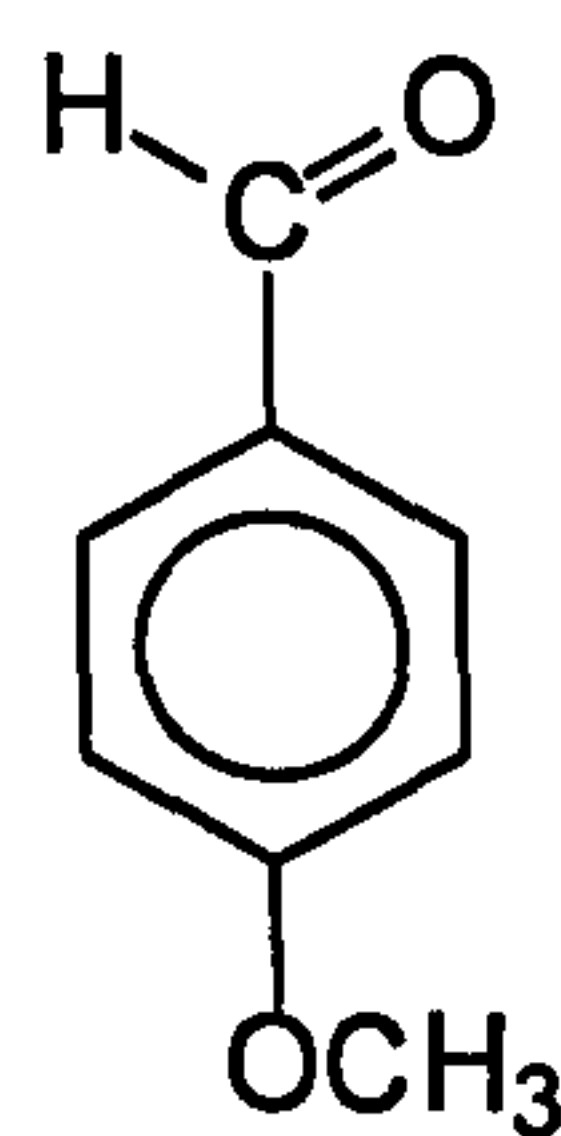
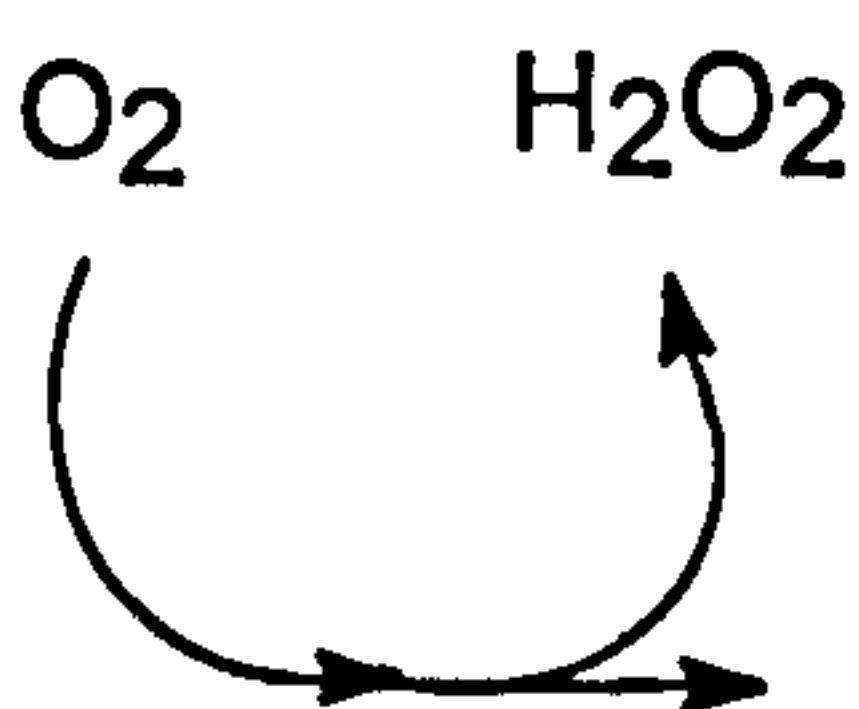
CINNAMYL ALCOHOL



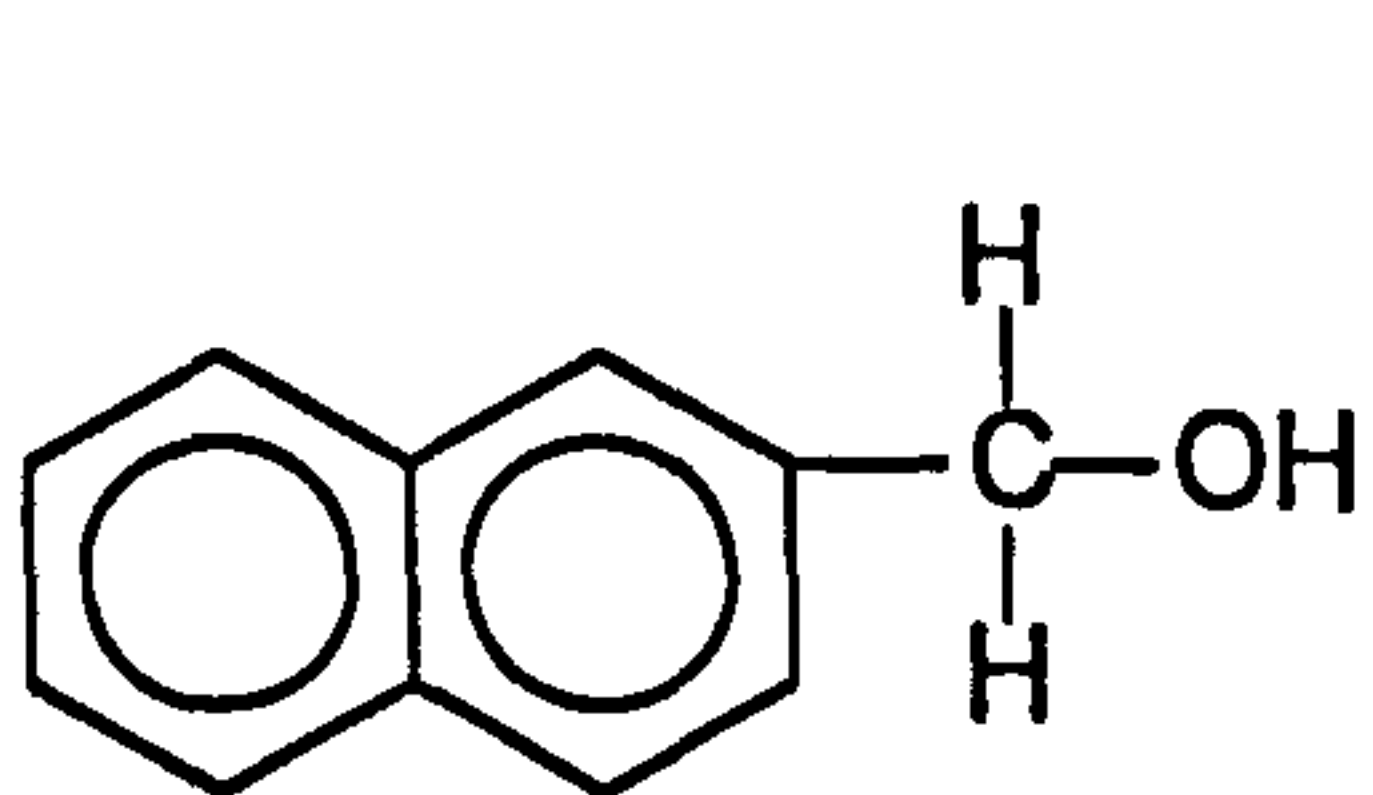
CINNAMALDEHYDE



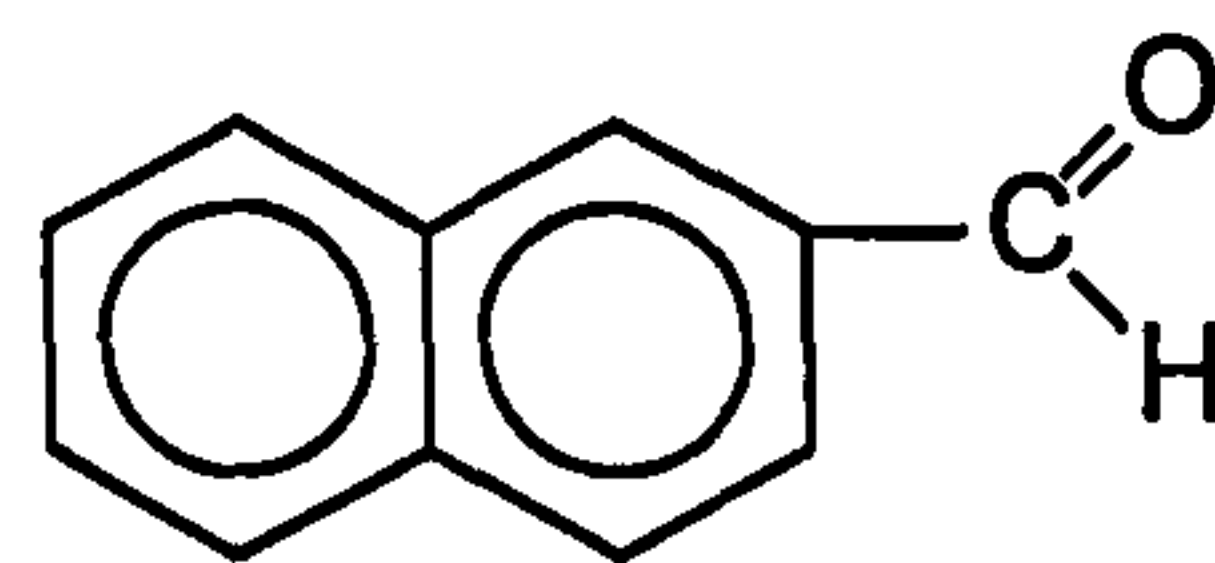
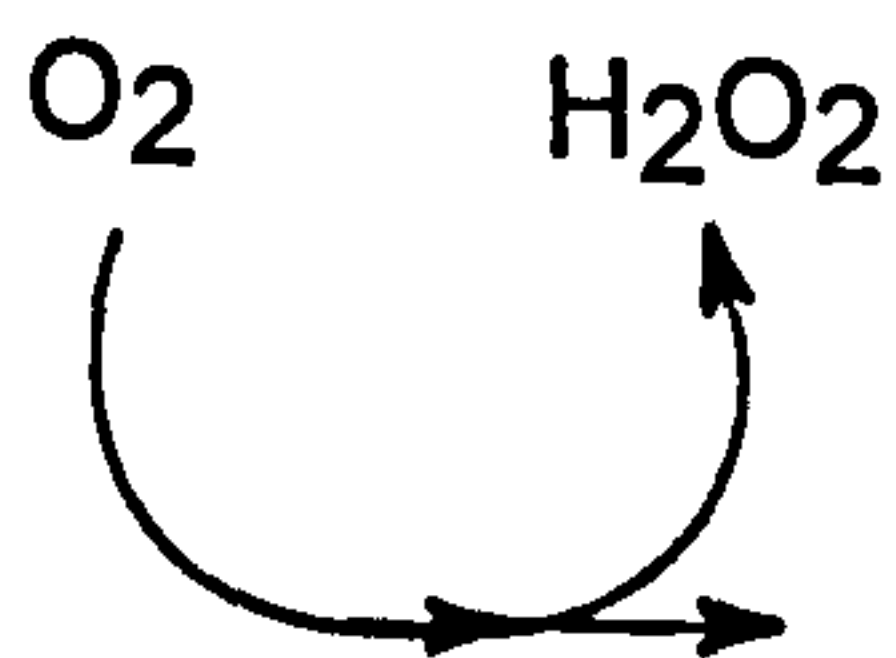
4-METHOXYBENZYL ALCOHOL



4-METHOXYBENZALDEHYDE



2-NAPHTHLENE-METHANOL



2-NAPHTHALDEHYDE

Figure 2.3.2.1.d) - The Oxidation of Aromatic Alcohols to their Corresponding Aldehydes by Aromatic Alcohol Oxidase

### 2.3.2.1.d) Direct Measurement of Aldehyde Production

Aromatic alcohol oxidase activity could be estimated by continuously following the production of aldehyde spectrophotometrically in the ultra-violet region of the spectrum (see figure 2.3.2.1.d)). This method could be used routinely but proved especially useful for stoichiometry estimations where aldehyde production was compared to hydrogen peroxide production. Potential substrates that interfered with peroxidase linked assays (by competing with TBHBA or *l*-DCF as substrates for horseradish peroxidase), for example coniferyl alcohol and 4-hydroxybenzyl alcohol, could be tested by measuring their conversion to their respective aldehydes. This method was also chosen for estimating activity with varying pH as the effect of pH on the dye and the linking enzyme (peroxidase) did not have to be considered.

The following aldehyde productions were followed :-

cinnamaldehyde (290nm,  $\epsilon = 26,200 \text{ M}^{-1} \text{ cm}^{-1}$ )

2-naphthaldehyde (250nm,  $\epsilon = 45,500 \text{ M}^{-1} \text{ cm}^{-1}$ )

4-methoxy-benzaldehyde (290nm,  $\epsilon = 15,000 \text{ M}^{-1} \text{ cm}^{-1}$ )\*

coniferaldehyde (342nm,  $\epsilon = 56,800 \text{ M}^{-1} \text{ cm}^{-1}$ )\*

4-hydroxybenzaldehyde (300nm,  $\epsilon = 8,800 \text{ M}^{-1} \text{ cm}^{-1}$ )\*

( \* from Farmer *et al* 1960)

For the routine estimation of activity the cuvette contained 50mM potassium phosphate buffer pH 7.4 and either 500 $\mu$ M cinnamyl alcohol (*cis/trans* mixed isomers) or 250 $\mu$ M 2-naphthalene-methanol. Quartz cuvettes were used and these were held in a thermostated cuvette holder at 25°C (*Arion*, *Limax* and *Helix*) or 30°C (*Achatina*). This method could be used to estimate the ratio of hydrogen peroxide to aldehyde produced by aromatic alcohol

oxidase when compared to the rates obtained by the TBHBA assay (continuous method). For such stoichiometric experiments an assay volume of 3ml contained 333 $\mu$ M cinnamyl alcohol, 33mM potassium phosphate buffer pH 7.4 and 0.1 mg ml<sup>-1</sup> catalase (for these experiments only to destroy hydrogen peroxide and so make the conditions as similar as possible to the TBHBA-AA peroxidase linked assay where peroxidase removes the hydrogen peroxide produced). The appearance of cinnamaldehyde was followed at 290nm ( $\epsilon$  = 26,200 M<sup>-1</sup> cm<sup>-1</sup>).

For the determination of activity with varying pH using particulate activity derived from *Arion ater* digestive gland the following buffers (all at 50mM) were used :- sodium citrate (pH 3.0 - 6.2), sodium cacodylate hydrochloride (pH 5.0 - 7.4), potassium phosphate (pH 6.0 - 8.0), tris-hydrochloride (pH 7.4 - 9.0), diethanolamine hydrochloride (pH 8.0 - 10.0). 2-Naphthalene-methanol (250 $\mu$ M) in totally aqueous phase was employed as the substrate. The appearance of 2-naphthaldehyde was monitored continuously at 250nm.

For the determination of activity with varying pH using solubilised activity derived from *Achatina fulica* digestive gland the following buffers were used :- potassium acetate (100mM, pH 3.0 - 5.5), potassium phosphate (100mM, 6.0 - 8.0), glycine-NaOH (66mM, pH 8.0 - 10.0). The substrate employed was cinnamyl alcohol (1mM) in totally aqueous phase. The appearance of cinnamaldehyde was monitored continuously at 290nm.

The absorbance of cinnamaldehyde and 2-naphthaldehyde was unaffected by the pH across the range used in these experiments.



### 2.3.2.1.e) Oxygen Consumption

Oxygen consumption was estimated by means of a Rank-type oxygen electrode. The electrode was calibrated with sodium dithionite (zero oxygen) and air-saturated distilled water (100% oxygen). The solubility of oxygen (from air) in distilled water was calculated as follows :-

$$[O_2] = \beta_T \times P \times F(O_2)$$

where  $\beta_T$  is the capacitance of oxygen in water at a given temperature (T) ( $\mu\text{mol dm}^{-3} \text{ mmHg}^{-1}$ ), P is the barometric pressure (mmHg) and  $F(O_2)$  is the fraction of oxygen in air (0.2095) (Dejours 1981). Experiments were at 22°C ( $\beta = 1.782 \mu\text{mol dm}^{-3} \text{ mmHg}^{-1}$ ) and 751 mmHg. Under these conditions the solubility of molecular oxygen from air in distilled water is 277  $\mu\text{M}$  (100%  $O_2$ ). The electrode response was directly proportional to the oxygen consumed.

The production of hydrogen peroxide was measured simultaneously using a modification of the TBHBA-AA peroxidase linked assay described previously (section 2.3.2.1.b)). This allowed the oxygen consumption to be directly compared to the production of hydrogen peroxide. The use of the peroxidase linked assay was also necessary to remove the hydrogen peroxide produced by converting it to water (see figure 2.2.3.3.b)); this prevented some of the consumed oxygen being released as the result of the spontaneous breakdown of hydrogen peroxide which would lead to serious inaccuracies.

The electrode chamber contained 0.2ml of enzyme sample (crude membrane preparation derived from Arion ater digestive gland), 25  $\mu\text{M}$  salicylaldehyde (to inhibit possible contaminating aldehyde oxidase activity), 0.2% w/v 3-amino-1,2,4-triazole (to

inhibit catalase), 1mM potassium cyanide (to inhibit oxygen consumption by mitochondrial membranes but could also potentially cause up to 60-70% inhibition of the peroxidase (Guibault *et al* 1968)), 1.0mg of TBHBA, 0.2mg of 4-aminoantipyrine, 0.05mg of horseradish peroxidase type II (in excess to ensure sufficient activity in the presence of cyanide) and 25mM potassium phosphate buffer pH 7.4 in a total volume of 5ml. The peroxidase was added last to ensure that the available cyanide inhibited mitochondrial cytochrome oxidase rather than peroxidase. 1ml of this was immediately withdrawn from the electrode chamber and kept on one side to act as a no-substrate control. 40 $\mu$ l of 56mM cinnamyl alcohol in methanol (to give a final concentration in the chamber of 0.56mM) was added to the electrode chamber through the narrow opening at the top with a syringe taking care not to add any air bubbles. The chamber contents were stirred continuously. The reaction was followed by monitoring oxygen consumption. When approximately 20% of the oxygen had been consumed an aliquot of 1ml was withdrawn and added to 2ml of 0.5M sodium citrate buffer pH 3.0 to stop the reaction. At this point 2ml of 0.5M sodium citrate buffer pH 3.0 was also added to the no-substrate control. Both were centrifuged at 3,000 r.p.m. for 20 minutes in a bench centrifuge and the absorbance of the test read against the no-substrate control ( $\epsilon = 29,000 \text{ M}^{-1} \text{ cm}^{-1}$  with respect to hydrogen peroxide). The oxygen consumed could be compared to the amount of hydrogen peroxide produced to give an estimate of the stoichiometry of the reaction.

Solubilised activity was unsuitable for this experiment because the detergent solution tended to trap air bubbles; there was also the possibility that the detergent would adversely affect the electrode membrane. Solubilised preparations were insufficiently active (due to being more dilute with respect to enzyme activity) to give a fast enough rate with the oxygen electrode.



### 2.3.2.2. Aldehyde Oxidase

Aldehyde oxidase activity could be routinely estimated by following the production of hydrogen peroxide using a slight modification of the methods previously described for mannitol oxidase (section 2.2.3.3.a) and b)).

For the 2,7 -*l* -dichlorofluorescein peroxidase linked assay 50µM of aldehyde or 0.7mM of hypoxanthine was substituted for the mannitol otherwise all reagents were as described in section 2.2.3.3.a). The cuvettes were held in thermostated cuvette holder at 15°C (*Arion*, *Limax* and *Helix*) or 30°C (mouse) and the absorbance monitored continuously at 502nm. Control assays lacking enzyme or substrate were also performed. Benzaldehyde at varying concentrations was employed as the substrate in kinetic studies.

The TBHBA-AA peroxidase linked method was only used when activity was derived from *Achatina fulica*. The assay conditions were essentially as described for mannitol oxidase (section 2.2.3.3b.)) except that 2mM or 0.2mM of the purine, pyrimidine or aldehyde to be tested was substituted for mannitol. The substrates were dissolved in 50mM imidazole pH 7.2 to give stock solutions of 5mM or 0.5mM. The stock solution of hypoxanthine was heated to 90°C to ensure its full solubilisation. Dodecanal was supplied as 0.2ml of a saturated aqueous solution added to the incubation to give a final volume of 0.5ml (final concentration of 40% saturation). Incubation was at 30°C. Suitable control assays lacking enzyme or substrate were also included. Several modifications were made when crude tissue homogenates were to be assayed using the TBHBA-AA peroxidase linked method. 0.2% w/v 3-amino-1,2,4-triazole was included in the assay reagent and the reaction was terminated by the addition of 0.5ml of 10% w/v S.D.S.. The tubes were centrifuged at 3,000 r.p.m. for 20 minutes in a bench centrifuge to remove precipitated protein and the absorbance measured at 510nm.

### 2.3.2.3. Mannitol Oxidase

Mannitol oxidase activity was estimated by the TBHBA-AA peroxidase linked method described previously (section 2.2.3.3.b)). Potential substrates were tested at 200mM and the tubes incubated at 23°C (*Arion*, *Limax* and *Helix*) or 30°C (*Achatina*).

### 2.3.2.4. Protein Assay

The protein assay described previously (section 2.2.3.2.) was unsuitable for the estimation of protein in detergent solutions. The method of Hartree (1972) was modified slightly to allow the estimation of protein in homogenates, membrane preparations and detergent solubilised preparations.

Protein was precipitated in ten volumes of ice-cold acetone (acidified with 0.16% v/v concentrated hydrochloric acid) and pelleted by centrifugation in a bench centrifuge at 3,000 r.p.m. for 20 minutes. The protein pellet was dissolved in 1% w/v S.D.S. with heating to 95°C. 1ml of this was added to 0.9ml of solution A (10% w/v sodium carbonate, 0.2% w/v sodium tartrate, 0.2% w/v sodium hydroxide) and heated to 50°C for 10 minutes. The tubes were then cooled to room temperature and 0.1 ml of solution B (2% w/v sodium tartrate, 0.4% w/v sodium hydroxide, 1% w/v copper sulphate) was added. After 10 minutes 3.0ml of solution C (1 part Folin-Ciocalteu reagent to 15 parts water) was added and the tubes again heated to 50°C for 10 minutes. An assay tube was also included using 1ml of 1% S.D.S. in place of the protein (no-protein blank). The tubes were then cooled to room temperature and the absorbance read at 650nm against the no-protein blank.

A calibration of 0 -100µg of protein (bovine serum albumin) was constructed using the same procedure on each occasion that the assay was performed.



### 2.3.3. Cellulose Acetate Electrophoresis

Aldehyde oxidase activity derived from the 100,000g x 2 hour supernatant of digestive gland or kidney (*Achatina fulica* only) homogenates was electrophoresed on cellulose acetate strips and the aldehyde oxidase was visualised with a specific dye via substrate-dependent oxidation.

Cellulose acetate foils (5cm x 7cm) were applied centrally with 100,000 g x 2 hour supernatant. These were electrophoresed for approximately 2 hours at 2.5mA per foil. The electrophoresis buffer had a pH of 6.0 and contained 0.8% w/v potassium dihydrogen phosphate and 0.25% w/v disodium hydrogen phosphate dodecahydrate.

After electrophoresis the foils were treated with a specific electron-acceptor dye-system containing 10% w/v polyvinyl alcohol, 0.013 mg ml<sup>-1</sup> phenazine methosulphate, 0.6 mg ml<sup>-1</sup> thiazoyl blue tetrazolium salt (MTT), 25mM potassium phosphate buffer pH 7.4 and either 2.5mM benzaldehyde or 2.5mM salicylaldehyde. A dye containing 50µl of dodecanal per 20ml of dye in place of benzaldehyde or salicylaldehyde was also made. The presence of aldehyde oxidase was indicated by the appearance of a purple insoluble dye-deposit. Foils were also treated with a control dye prepared as above but lacking substrate to insure that any reaction observed was aldehyde dependent. The stained foils were scanned using a recording densitometer.

#### 2.3.4. Estimation of Apparent $K_m$ Values

An initial experiment was performed to give a rough estimate of the apparent  $K_m$ . Subsequent experiments used a lowest substrate concentration  $[s]$  of between one half and one times the approximate  $K_m$  value obtained above and a highest substrate concentration of approximately five times this value. Apparent  $K_m$  values were estimated by the Direct Linear Plot of Eisenthal and Cornish-Bowden (1974). Experimental data was processed using computer software designed by Dr. Paul Kirk (University of Wolverhampton) in which the  $K_m$  is estimated by taking the median (rather than the mean) of the intercepts as described by Cornish-Bowden and Eisenthal (1974).

## 3. RESULTS

## 3.1. Subcellular Fractionation Studies

### 3.1.1. Fractionation of *Arion ater* Digestive Gland

#### 3.1.1.1. Density-dependent Banding

*Arion ater* digestive gland P.N.S. was subjected to isopycnic centrifugation in a sucrose density gradient using a BXIV zonal rotor with a B29 insert (see section 2.2.2.1.). Figure 3.1.1.1. shows the enzyme distribution obtained in typical experiment. Several potential lysosomal markers were analysed. Aryl sulphatase appears to be the best marker for the lysosomes although much of the activity remains in the sample band (the first large volume fraction to be unloaded). This "soluble" activity is possibly due to the disruption of lysosomes during homogenisation and subsequent handling of the P.N.S.. The particulate activity is distributed at the dense end of the gradient with a pronounced peak at a density of  $1.26 \text{ g cm}^{-3}$ . Unlike the other potential lysosomal markers tested there is very little activity associated with lower density membranes.  $\beta$ -Glucuronidase is another potential lysosomal marker and again the bulk of the activity is found in the sample band. It also peaks at  $1.26 \text{ g cm}^{-3}$  but there is some activity associated with a lower density membrane most likely endoplasmic reticulum. Acid phosphatase (fluoride-sensitive  $\alpha$ -naphthyl phosphate hydrolase pH 5.0) is also associated with the lysosomes (peaking at  $1.26 \text{ g cm}^{-3}$ ) but there is a significant peak in the middle of the gradient that coincides with the peak of NADPH-cytochrome c reductase (marker for endoplasmic reticulum). Again there is significant "soluble" activity.

Sedimentable catalase is a marker enzyme for peroxisomes (Beaufay *et al* 1964). The distribution of this activity indicated that peroxisomes band at relatively high density ( $1.23 \text{ g cm}^{-3}$ ) and can clearly be distinguished from mitochondria (which peaks strongly at  $1.18 \text{ g cm}^{-3}$ , succinate dehydrogenase) and



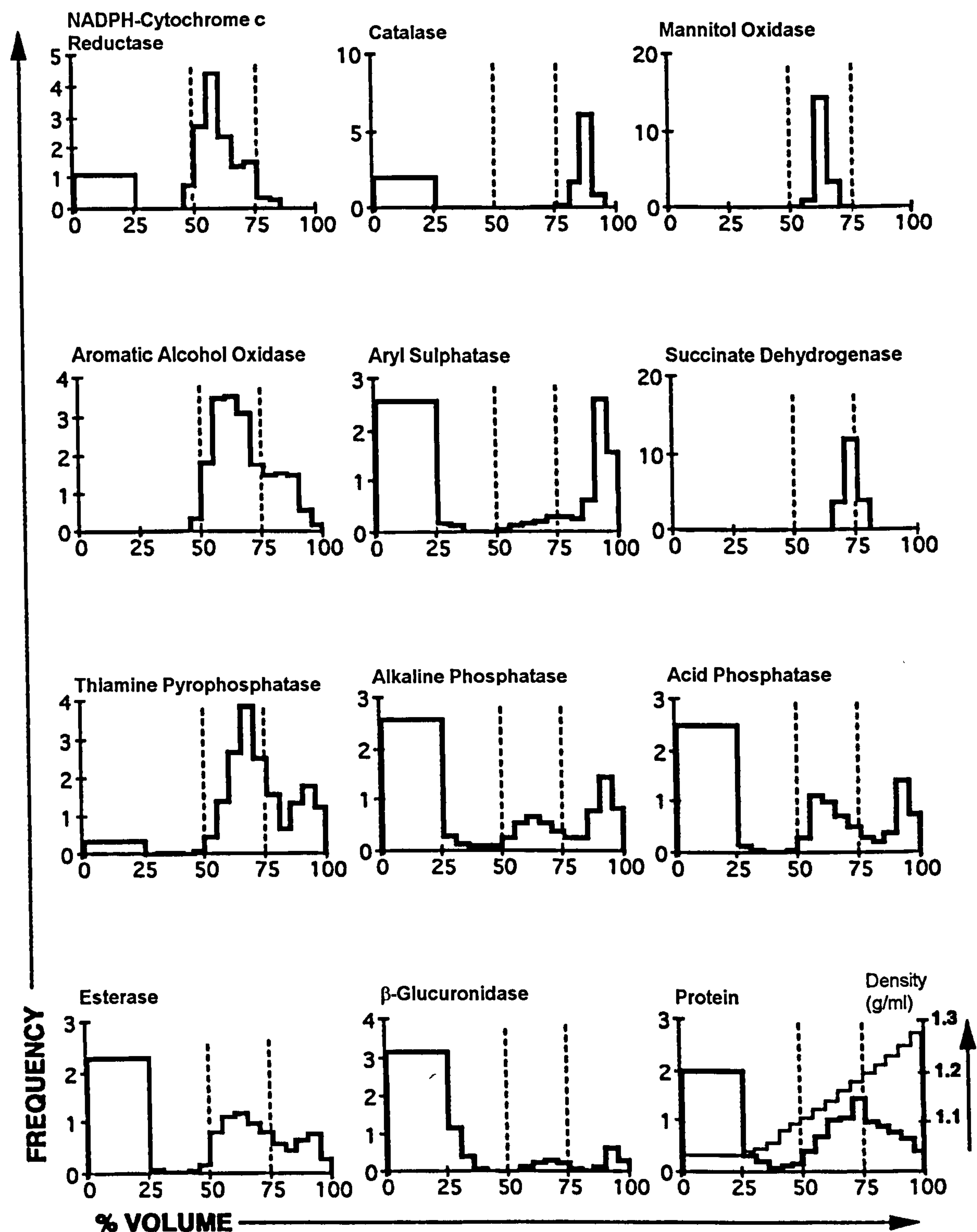


Figure 3.1.1.1. The Distribution of Enzyme Activities Following Sucrose Gradient Isopycnic Centrifugation (45,000 r.p.m. for 2.5 hours) of *Arion ater* Digestive Gland. The dotted lines enclose the middle part of the gradient.

lysosomes (peaking at  $1.26 \text{ g cm}^{-3}$ , aryl sulphatase). Approximately 50% of the activity is particulate; it is difficult to say with any certainty whether the remaining soluble activity is a genuine and significant cytosolic catalase activity or the result of damage to the peroxisomes during homogenisation causing the release of catalase.

Esterase ( $\alpha$ -naphthyl acetate hydrolase) is widely distributed; the peak of the activity is in the middle of the gradient (at a density of  $1.15 \text{ g cm}^{-3}$ ) and indicates the position of the endoplasmic reticulum. However there is also a second peak at a density of  $1.26 \text{ g cm}^{-3}$  possibly due to lysosomal proteases acting on the esterase substrate. This somewhat limits the use of esterase as a specific marker for the endoplasmic reticulum. NADPH-cytochrome c reductase is another potential marker for the endoplasmic reticulum (Beaufay *et al* 1974b). Surprisingly there is apparent "soluble" activity but the bulk of the activity is particulate peaking at a density of  $1.13 \text{ g cm}^{-3}$ . There is no apparent cyanide-insensitive activity associated with the mitochondria. This activity therefore appears to be a satisfactory marker for the endoplasmic reticulum in this tissue.

Thiamine pyrophosphatase has been identified as a marker for Golgi apparatus in mammals (Essner and Novikoff 1962) and gastropod molluscs (Ovtracht *et al* 1969) although activity is also to some extent associated with endoplasmic reticulum and plasma membrane in some tissues and this may limit its use as a specific marker for Golgi apparatus (Morré 1971). Apparent thiamine pyrophosphatase activity has a spread-out distribution peaking at a density of  $1.17 \text{ g cm}^{-3}$ . There is a second peak of activity associated with the lysosomal fractions at the dense end of the gradient most probably due to fluoride- and EDTA-insensitive non-specific phosphatases and pyrophosphatases. This further limits the use of this marker. Alkaline phosphatase, an enzyme activity associated with the plasma membrane, also has a rather spread-out distribution peaking at a density of  $1.15 \text{ g cm}^{-3}$ . There is also significant fluoride-insensitive activity found in the lysosomal fractions (density =  $1.26 \text{ g cm}^{-3}$ ). As

with thiamine pyrophosphatase the efficacy of this activity as a specific marker is limited by the presence of lysosomal non-specific phosphatases.

Aromatic alcohol oxidase activity peaks at a density of 1.13 - 1.15 g cm<sup>-3</sup> and has a spread-out distribution. The distribution is very similar to that of NADPH-cytochrome c reductase up to a density of 1.18 g cm<sup>-3</sup>. However NADPH-cytochrome c reductase tails off sharply above this density whereas aromatic alcohol oxidase activity continues up to a density of at least 1.23 g cm<sup>-3</sup> possibly indicating that a greater proportion of this activity is associated with rough endoplasmic reticulum. Aromatic alcohol oxidase and particulate esterase have very similar distributions if the apparent esterase activity attributable to lysosomes is ignored. No soluble aromatic alcohol oxidase activity is observed provided precautions are taken to eliminate cytosolic aldehyde oxidase (see section 2.2.3.4.). The results indicate that aromatic alcohol oxidase is most probably associated with the endoplasmic reticulum.

Mannitol oxidase activity is localised in a relatively low density membrane (density = 1.15 g cm<sup>-3</sup>) and is very well resolved into a small volume of the gradient. This membrane can be clearly distinguished from mitochondria (succinate dehydrogenase, peaking at 1.18 g cm<sup>-3</sup>), peroxisomes (catalase, peaking at 1.23 g cm<sup>-3</sup>) and lysosomes (aryl sulphatase, peaking at 1.26 g cm<sup>-3</sup>). Other organelles that peak in the middle part of the gradient include plasma membrane (alkaline phosphatase), Golgi apparatus (thiamine pyrophosphatase) and endoplasmic reticulum (NADPH-cytochrome c reductase, esterase and aromatic alcohol oxidase). However all of these organelles have a spread-out distribution which is completely different to the sharply peaked distribution of mannitol oxidase. This indicates that mannitol oxidase is not associated with any of the above organelles but rather with a quite distinct low-density membrane structure.

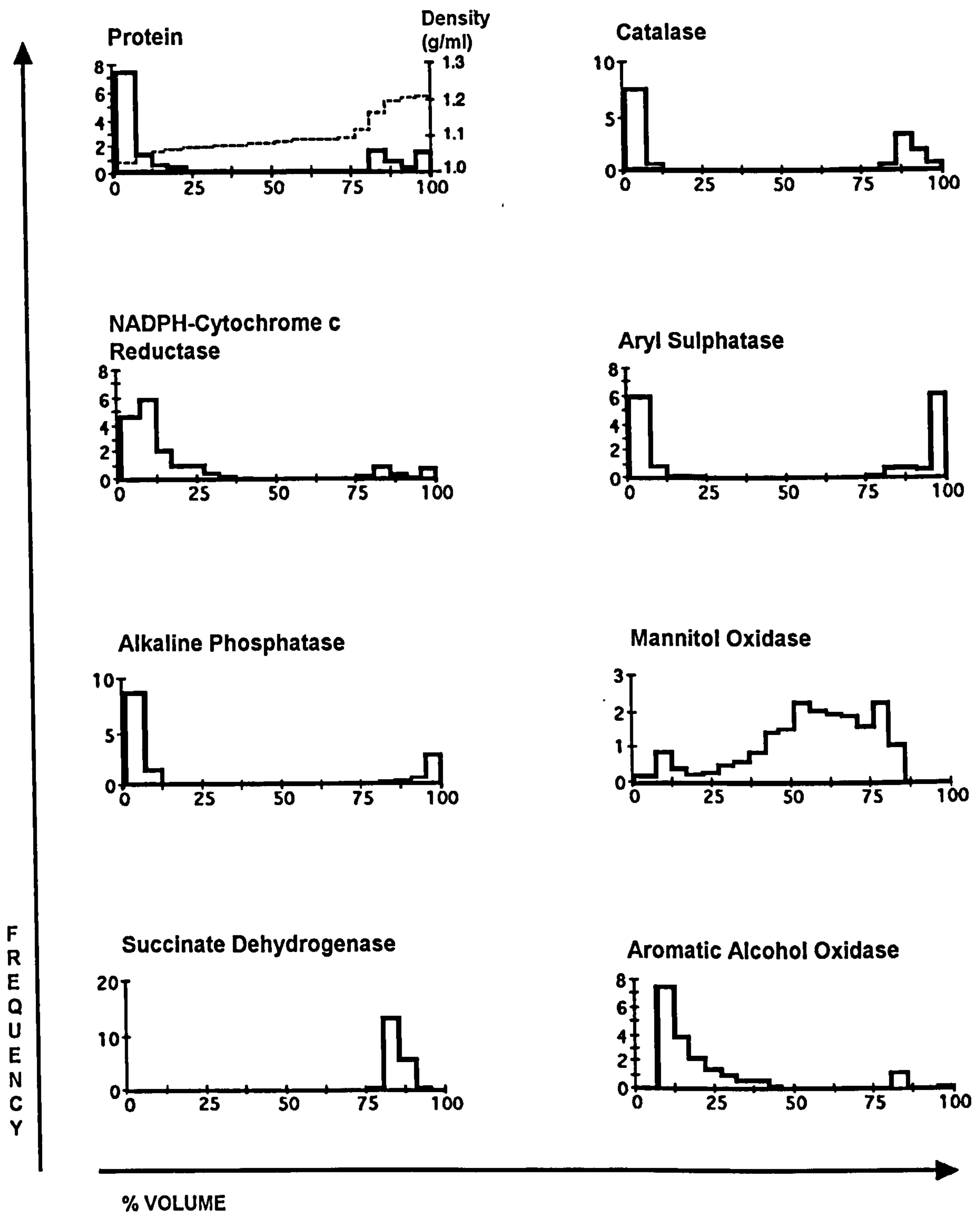


Figure 3.1.1.2.a) Distribution of enzymes in a shallow sucrose gradient (resting on a dense cushion) after rate-dependent sedimentation (16,000 r.p.m. for 30 minutes) of Arion ater digestive gland P.N.S. in a BXIV zonal rotor.



### 3.1.1.2. Rate -dependent Banding

*Arion ater* digestive gland P.N.S. was subjected to rate-dependent sedimentation in a shallow sucrose gradient (12 - 28% resting on a dense cushion) using a Beckman or M.S.E. BXIV zonal rotor; centrifugation was at 16,000 r.p.m. for 30 minutes (see section 2.2.2.3.). The results of a typical fractionation are shown in figure 3.1.1.2.a). Lysosomes (aryl sulphatase), peroxisomes (sedimentable catalase) and mitochondria (succinate dehydrogenase) have all sedimented quickly. They have reached the end of the gradient and penetrated the cushion to varying extents. The endoplasmic reticulum marker NADPH-cytochrome reductase has remained predominately in the sample band and has barely entered the gradient. Apart from the apparent "soluble" activity the distribution of this marker is the same as that of aromatic alcohol oxidase supporting the conclusion that aromatic alcohol oxidase is associated with the endoplasmic reticulum. Alkaline phosphatase attributable to plasma membrane (rather than lysosomes) remains almost entirely in the sample band and has not sedimented at all. In contrast mannitol oxidase carrying structures have sedimented well into the gradient but have not reached the cushion. Mannitol oxidase is both well separated from all the other markers and is associated with only a small proportion of the total protein. Mitochondrial (succinate dehydrogenase), lysosomal (aryl sulphatase) and endoplasmic reticulum (aromatic alcohol oxidase) enriched fractions were examined by electron microscopy (figures 3.1.1.2. b), c) and d)). The results confirmed the biochemical analysis.

In rate-dependent banding experiments (such as that shown in figure 3.1.1.2.a)) mannitol oxidase bearing membranes have sedimented into the gradient and have separated from the slow-moving microsomes (endoplasmic reticulum etc.). This is consistent with the observation of Vorhaben *et al* (1980) that mannitol oxidase carrying structures in *Helix aspersa* digestive gland were fast sedimenting and pelleted into mitochondrial fractions in differential pelleting experiments.



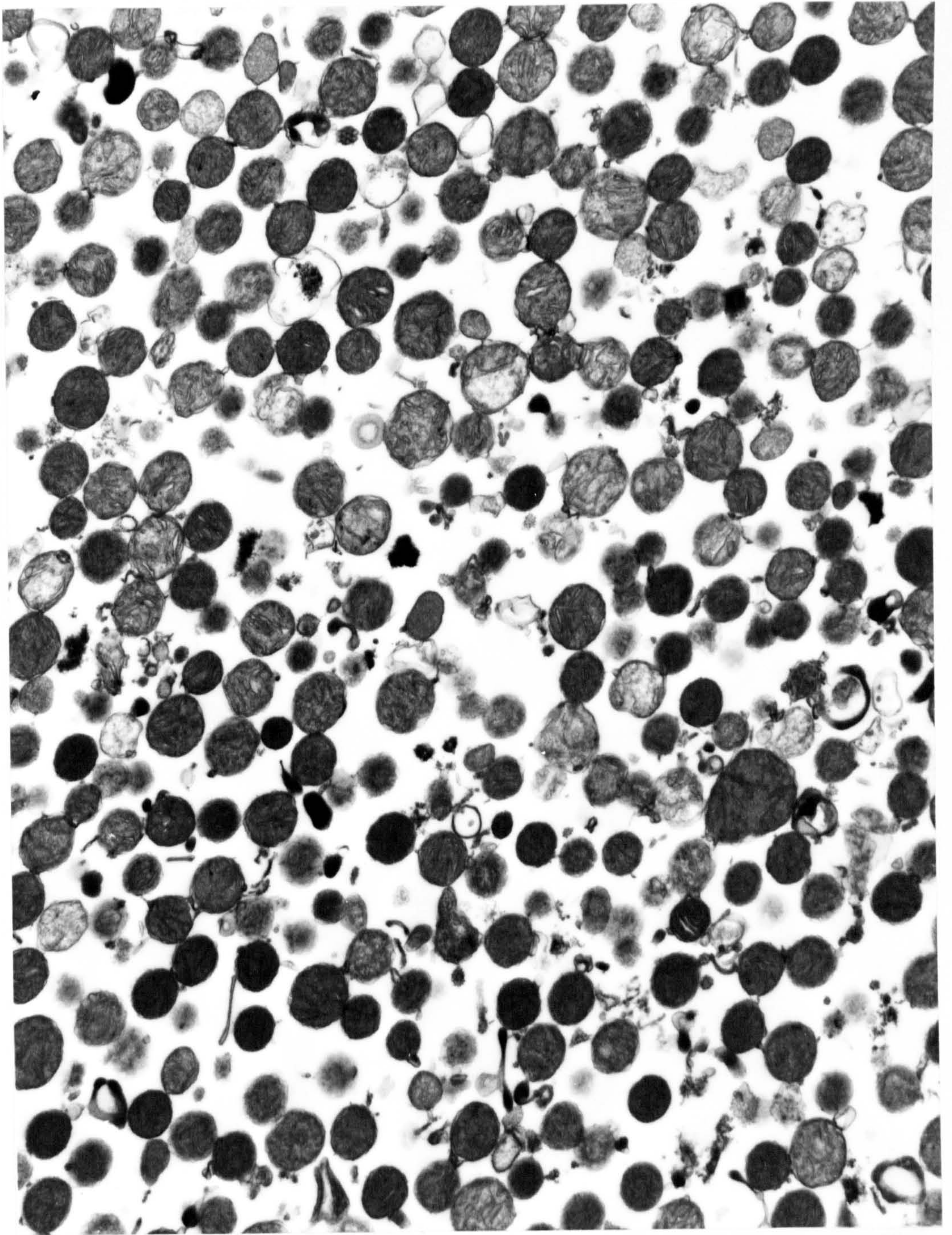


Figure 3.1.1.2. b) - Electron micrograph of a pellet prepared from mitochondrion enriched fractions from a rate separation such as that shown in figure 3.1.1.2.a). (x 14,000).



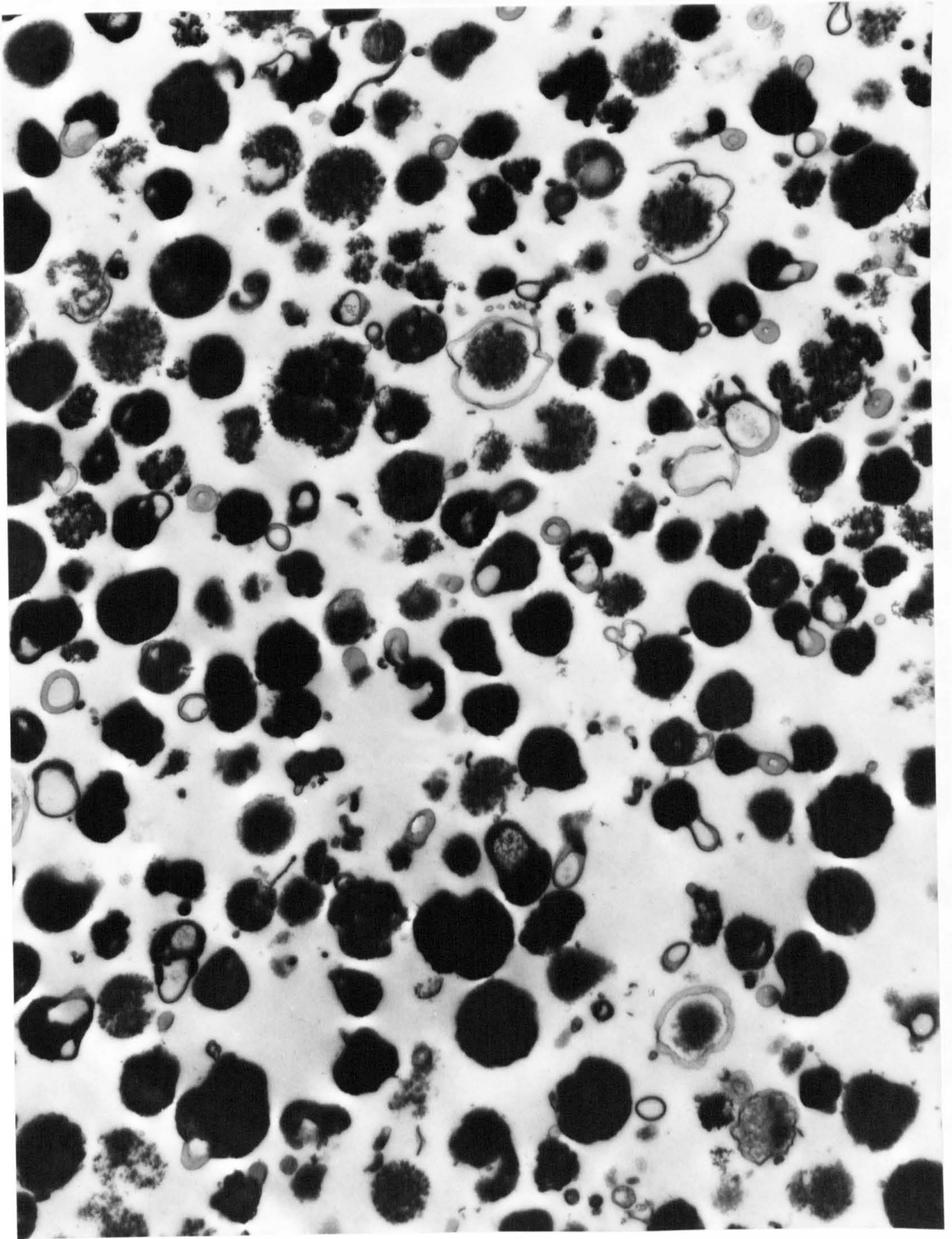


Figure 3.1.1.2. c) - Electron micrograph of a pellet prepared from lysosome enriched fractions from a rate separation such as that shown in figure 3.1.1.2.a). (x 14,000).



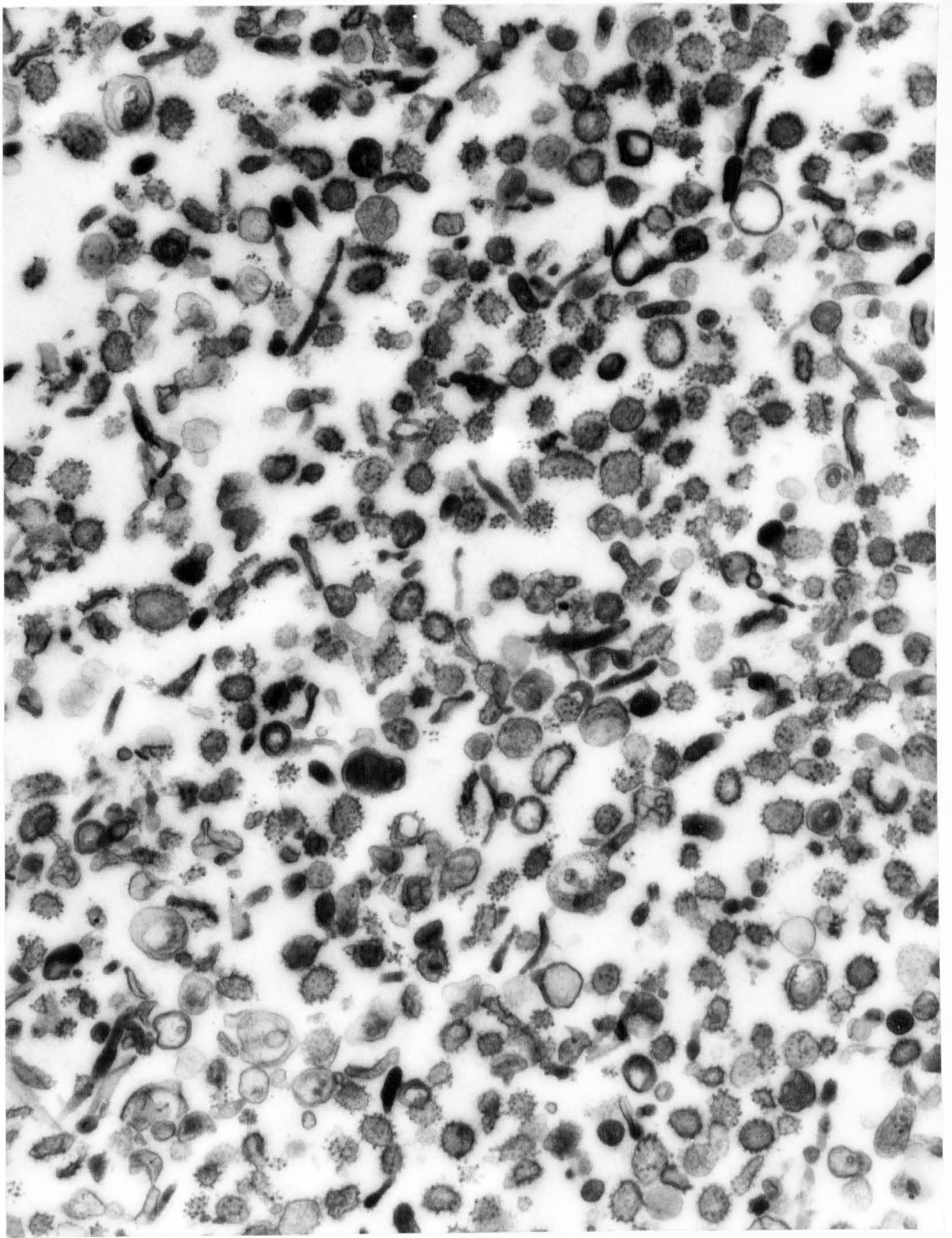


Figure 3.1.1.2. d) - Electron micrograph of a pellet prepared from endoplasmic reticulum enriched fractions from a rate separation such as that shown in figure 3.1.1.2.a). (x 38,000).



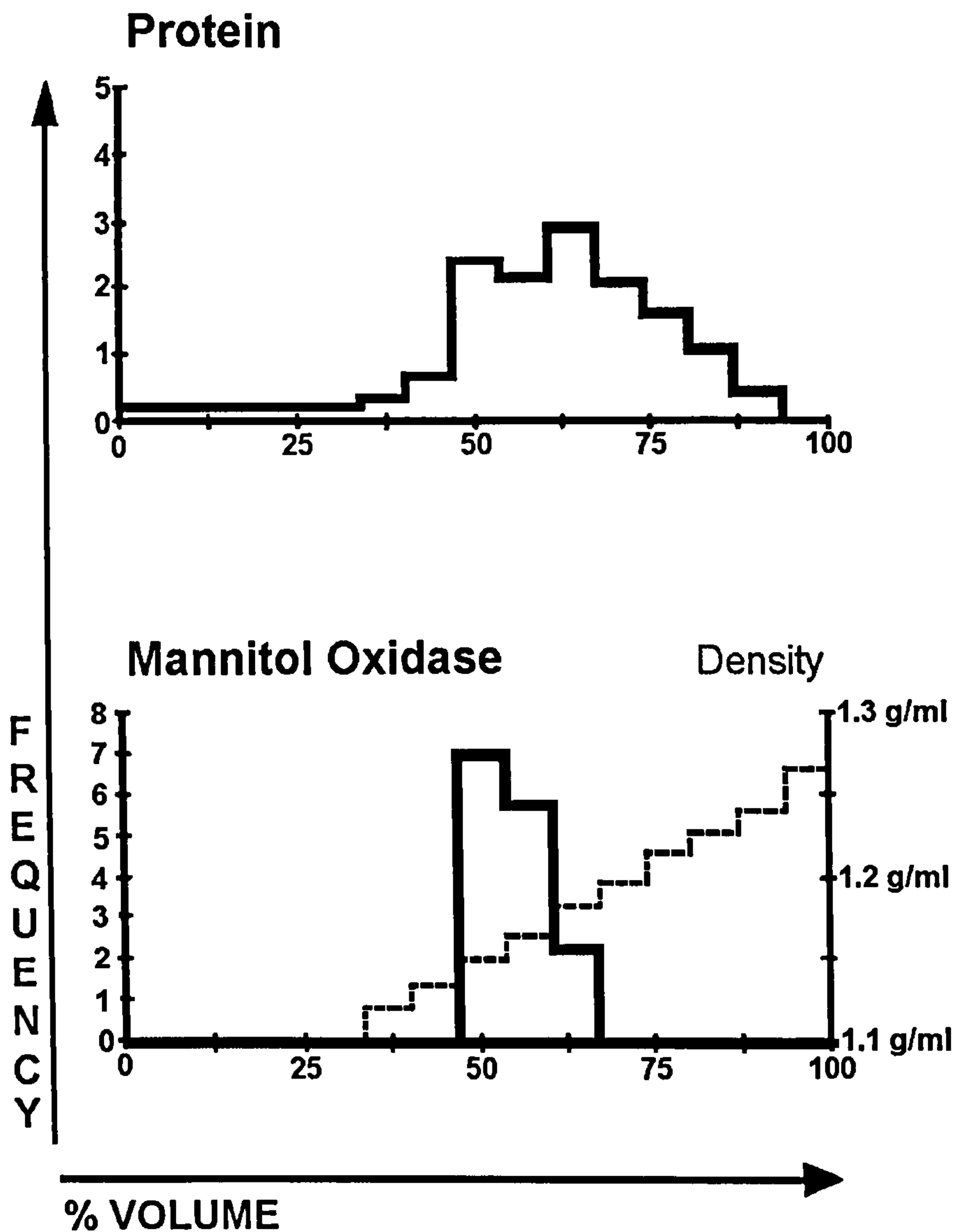


Figure 3.1.1.3.a) The distribution of mannitol oxidase and protein in a sucrose gradient after isopycnic centrifugation (45,000 r.p.m. for 2 hours) of mannitol oxidase enriched fractions taken from a rate-gradient (see section 3.1.1.2.). Mannitol oxidase was enriched approximately 50-fold with respect to the original P.N.S. in the peak fractions.

### 3.1.1.3. Dual Gradient Method for the Isolation of Mannitol Oxidase Carrying Structures and their Identification

*Arion ater* digestive P.N.S. was subjected to rate-dependent sedimentation as described in the previous section. The peak mannitol oxidase fractions from such experiments were subsequently subjected to isopycnic centrifugation in a sucrose density gradient using a BXIV zonal rotor with a B29 insert (see section 2.2.2.4.). This enabled reasonably pure and intact mannitol oxidase carrying structures to be obtained.

The distributions of mannitol oxidase and protein in such an isopycnic gradient are shown in figure 3.1.1.3.a). Mannitol oxidase was enriched approximately 50-fold in the peak fractions compared to the original P.N.S.. Mannitol oxidase peaks strongly at  $1.15 \text{ g cm}^{-3}$ . Protein also peaks at this density but there is a second peak occurring at a higher density ( $1.18 \text{ g cm}^{-3}$ ). Although succinate dehydrogenase was not readily detectable in this gradient this second protein peak almost certainly corresponds to mitochondrial contamination as it is at the density at which the mitochondria band in sucrose isopycnic gradients (see section 3.1.1.1.).

S.D.S.-P.A.G.E. analysis of the mannitol oxidase-enriched fractions (figure 3.1.1.3.b)) revealed the presence of a predominant polypeptide at 68 - 70 kDa with fainter bands at 58 - 60 kDa. When compared to mitochondrial enriched fractions (derived from a rate-dependent gradient as described in section 3.1.1.2.) these latter bands corresponded to the most abundant mitochondrial polypeptides. This confirms the view that the second protein peak found in the gradient was due to mitochondrial contamination. Vorhaben *et al* (1986) reported that S.D.S.-P.A.G.E. analysis of a partially purified and solubilised mannitol oxidase preparation exhibited major polypeptides of high molecular mass and 68 kDa with minor



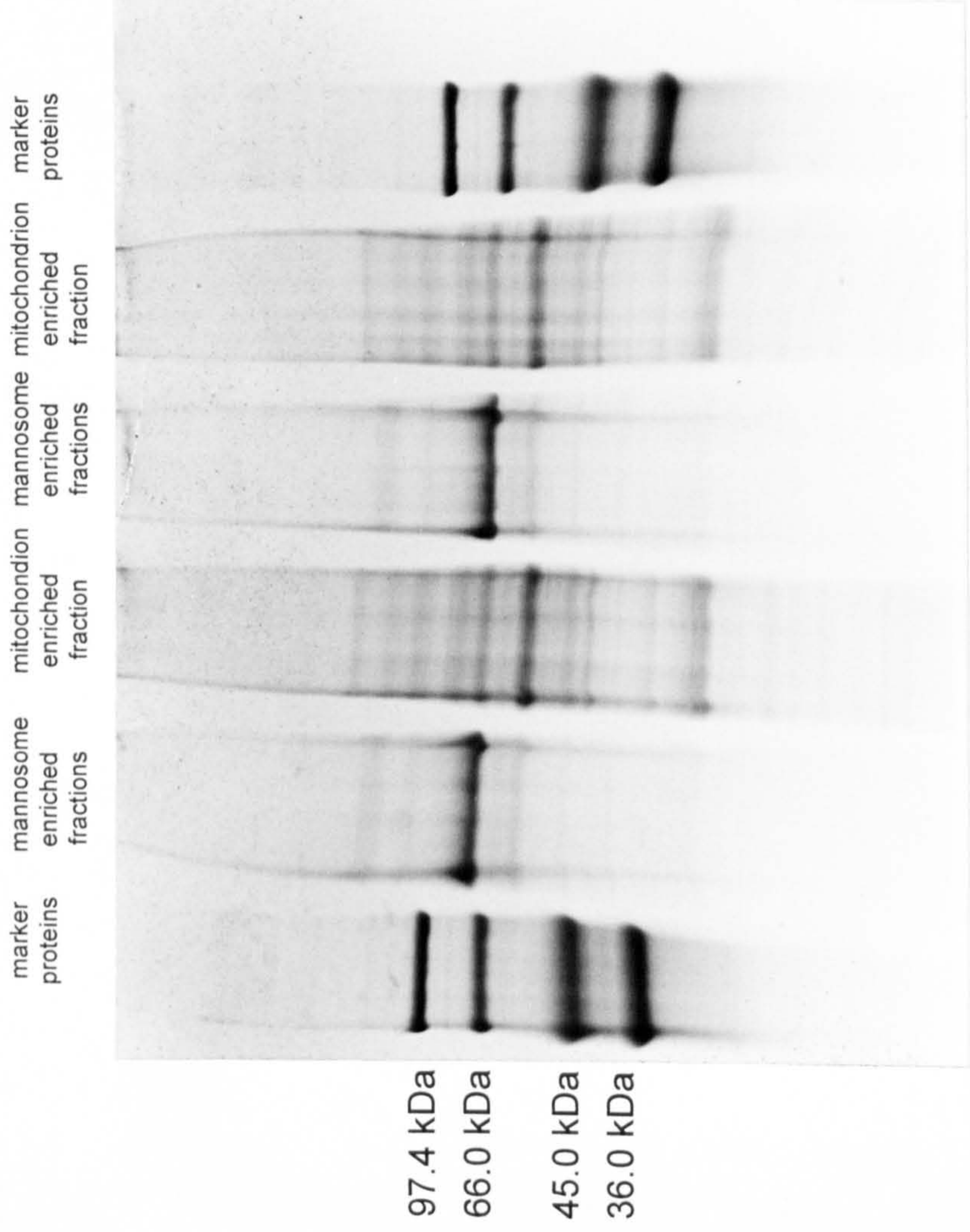


Figure 3.1.1.3.b) - SDS-PAGE analysis on a 12% gel of mannitol oxidase enriched fractions and mitochondrion enriched fractions.

The marker proteins are phosphorylase b (97.4 kDa), bovine albumin (66.0 kDa), egg albumin (45.0 kDa) and glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa). The mitochondrion enriched fraction was derived from an experiment such as that described in section 3.1.1.2.. The mannosome enriched sample was derived from an experiment such as that shown in figure 3.1.1.3.a).



bands also observed between 45 and 68 kDa. This pattern is very similar to that observed for the isolated organelles.

Electron microscopic examination of the of the peak mannitol oxidase fractions (figure 3.1.1.3.c)) showed the predominant presence of unusual tubular structures. In transverse section these structures commonly had a hexagonal arrangement of tubules surrounding a central tubule and were enclosed by a smooth membrane. The tubules were typically of approximately 40 - 60 nm in diameter. Moya and Rallo (1975) reported identical structures to these in the digestive gland of the slug *Arion empiricorum* (syn. *A. ater*). These cytoplasmic structures were described as intracisternal polycylinders (I.C.P.C.) and consisted of four tubules arranged rhomboidally or seven tubules arranged hexagonally around a central tubule with a surrounding membrane. The diameter of the tubules was estimated to be around 50nm ( $\pm$  10nm).

Structures of the same appearance and dimensions have been described by electron microscopists in the digestive glands of the gastropods *Arion rufus* (syn. *A. ater*, *A. empiricorum*) (Czarna et al 1985), *Arion hortensis* (David and Götze 1963), *Arion lusitanicus* (Triebkorn and Köhler 1992), *Theba pisana* (Cornejo 1987) and *Helix pomatia* (Abolinš-Krogis 1970). The same structures have also been reported in the intestine of the slug *Deroceras reticulatum* fed a lethal dose of the molluscicide cloethocarb (Triebkorn and Künast 1990).

The interim term "mannosome" has been proposed for these tubular structures associated with mannitol oxidase in *Arion ater*.



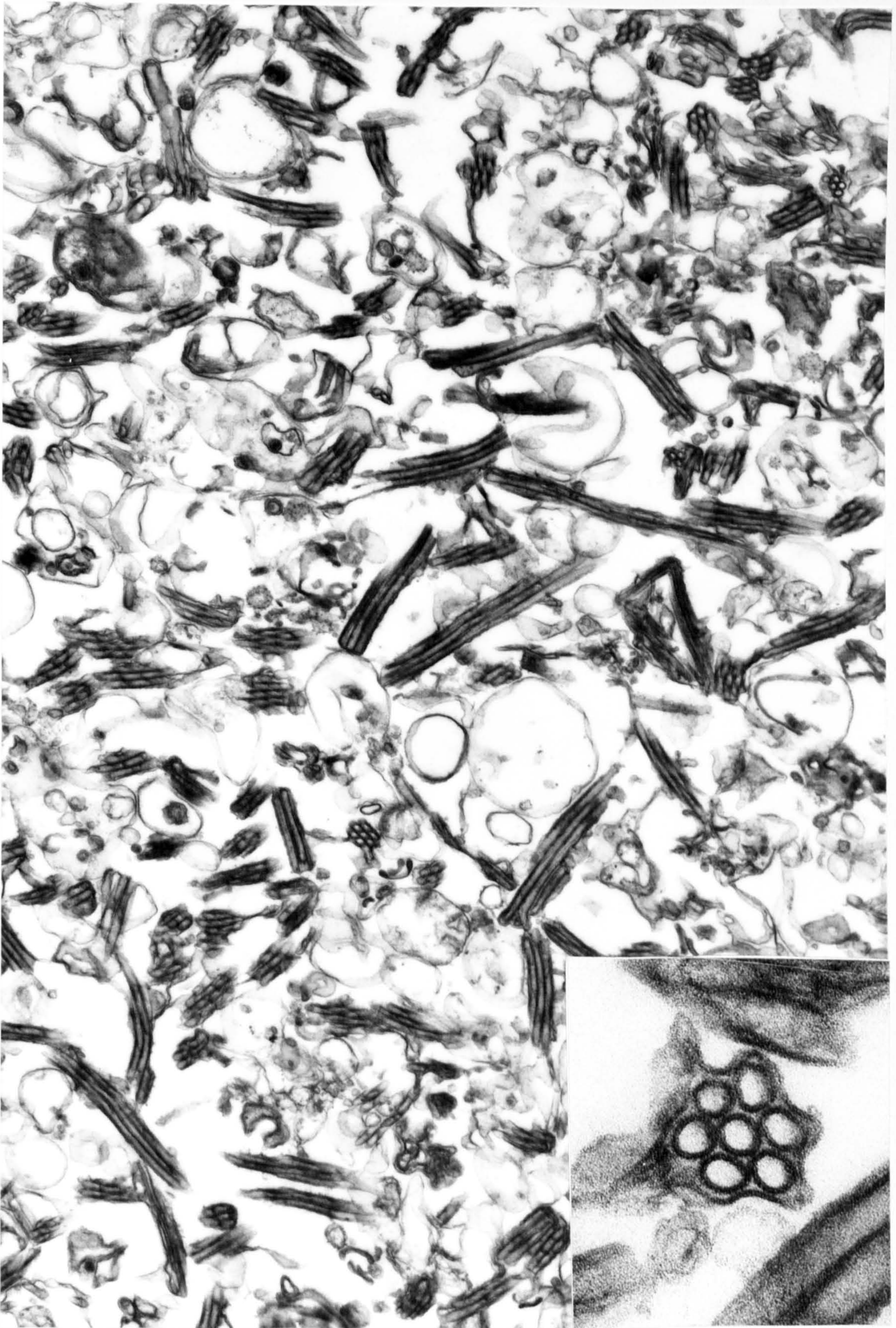


Figure 3.1.1.3.c) - Electron micrograph of a pellet prepared from mannitol oxidase enriched fractions of an isopycnic separation (such as that shown in figure 3.1.1.3.a)) of peak mannitol oxidase fractions from a rate gradient. Note the numerous tubular structures (x 30,000). Inset :- Hexagonal arrangement of tubules seen in tranverse section (x 150,000).



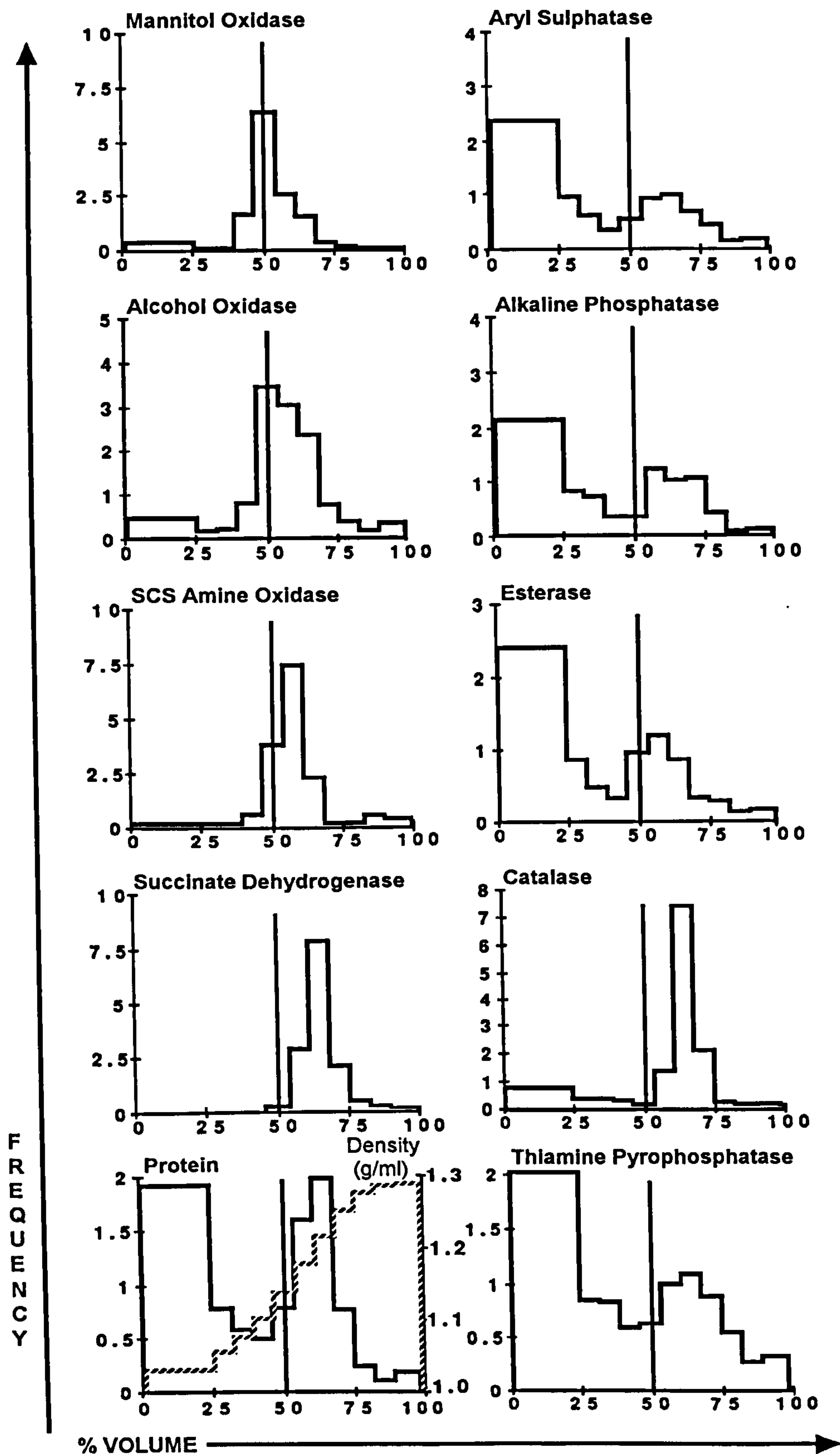


Figure 3.1.2.1. The distribution of enzyme activities following sucrose gradient isopycnic centrifugation (45,000 r.p.m. for 2.5 hours) of *Achatina fulica* digestive gland P.N.S..



### 3.1.2. Fractionation of *Achatina fulica* Digestive Gland P.N.S.

#### 3.1.2.1. Density-dependent Banding

This large african species was investigated because large quantities of tissue could be obtained rapidly without dissecting a large number of animals. *Achatina fulica* digestive gland P.N.S. was subjected to isopycnic centrifugation (45,000 r.p.m. for 2.5 hours) in a sucrose density gradient using a Beckman BXIV zonal rotor with a B29 insert (see section 2.2.2.2.). Figure 3.1.2.1. illustrates the distribution of enzyme activities in a typical experiment.

The lysosomal marker aryl sulphatase has significant "soluble" activity. The particulate activity peaks at a density of  $1.21 \text{ g cm}^{-3}$  indicating that the lysosomes in this species may not be as dense as those found in *Arion ater* digestive gland (which peak at a density of  $1.26 \text{ g cm}^{-3}$ ). It is likely that the lysosomes have fully equilibrated to their respective densities as the  $g \times \text{time}$  is very high (45,000 r.p.m. for 2.5 hours). Succinate dehydrogenase (mitochondrial marker) and particulate catalase (peroxisomal marker) also peak at a density of  $1.21 \text{ g cm}^{-3}$  although both of these activities peak sharply compared to aryl sulphatase. The mitochondria and peroxisomes do not appear to be as readily separated in this species as they are in *Arion ater* although this could be due the lower resolution obtained by collecting relatively fewer fractions. Thiamine pyrophosphatase (a Golgi membrane marker that has some limitations, see section 3.1.1.1.) is very widely distributed and may not be truly representative of the distribution of golgi membranes as it peaks in the same fraction as the lysosomes (marker aryl sulphatase, density =  $1.21 \text{ g cm}^{-3}$ ) where the interference of non-specific phosphatases and pyrophosphatases will be the greatest. Esterase peaks at a density of  $1.17 \text{ g cm}^{-3}$ . Again this distribution may not be truly

representative of the endoplasmic reticulum as there is a possibility of interference from apparent lysosomal esterase activity (which was significant in *Arion ater*). Alkaline phosphatase (plasma membrane marker) also peaks at a density of  $1.17 \text{ g cm}^{-3}$  but this marker also has limitations because of the probable interference of lysosomal phosphatases. Semicarbazide-sensitive (SCS) amine oxidase peaks quite strongly at a density of  $1.17 \text{ g cm}^{-3}$  and no "soluble" activity is observable. This enzyme activity is clearly not located in either mitochondria or peroxisomes as it has been well separated from the markers for these organelles. Plasma membrane would seem to be a likely candidate as the organelle bearing this activity as SCS-amine oxidase peaks in the same fraction as alkaline phosphatase. Evidence from mammalian tissue supports this localisation (Barrand and Callingham 1984). It is also conceivable that SCS-amine oxidase and alkaline phosphatase would have very similar distributions but for the interference of soluble and lysosomal phosphatase activity.

Alcohol oxidase (crotyl alcohol oxidase) and mannitol oxidase both peak at a density of  $1.15 \text{ g cm}^{-3}$  with no appreciable "soluble" activity. Alcohol oxidase is almost certainly localised in the endoplasmic reticulum and this activity is probably the most representative of the distribution of this organelle. Mannitol oxidase is fairly sharply resolved into a small volume of the gradient but not to the extent observed with *Arion ater* (see section 3.1.1.1.); its distribution is overlapped by that of alcohol oxidase. The mannitol oxidase bearing membranes are not clearly distinguishable from the endoplasmic reticulum by isopycnic centrifugation as was the case in *Arion ater*. One explanation could be that the "mannosomes" in *Achatina fulica* respond somewhat differently to homogenisation. Alternatively it is possible that the highly structured "mannosomes" observed in *Arion ater* are not present in this species or that the mannitol oxidase bearing membranes in the cell are not completely separate from the endoplasmic reticulum.

### 3.1.3. Comparative Analytical Fractionation of Digestive Gland P.N.S. Derived from Several Species of Gastropod Mollusc

#### 3.1.3.1. Density-dependent Banding

Digestive gland P.N.S. from several species of gastropod mollusc was subjected to rapid small-scale density-dependent (isopycnic) banding experiments using a Beckman Ti-50 vertical rotor (45,000 r.p.m. for 2 hours) (see section 2.2.2.5.). The results of the fractionation of digestive gland P.N.S. from *Arion lusitanicus*, *Limax flavus* and *Helix aspersa* were compared to the results for *Arion ater* obtained by the same method. These experiments together with rate dependent-banding experiments (see next section) were performed so as to investigate the localisation of mannitol oxidase and to provide centrifugal evidence for the presence of "mannosomes" in other species of gastropod mollusc in addition to *Arion ater*.

The results of typical isopycnic banding experiments are shown in figures 3.1.3.1.a) *Arion ater*, b) *Arion lusitanicus*, c) *Limax flavus* and d) *Helix aspersa*. In all four species the mitochondria (marker succinate dehydrogenase) peak sharply at a density of  $\sim 1.18 \text{ g cm}^{-3}$ . In all cases the catalase activity is mostly soluble but has begun to sediment due to its large size. Particulate catalase (peroxisomes) bands at higher density than the mitochondria. The lysosomal marker aryl sulphatase (results not shown) is almost completely soluble with very little particulate activity being observed. The very small proportion of the activity that was particulate sedimented to the dense end of the gradient in all four species.

Aromatic alcohol oxidase was chosen as the marker for the endoplasmic reticulum as this localisation had been indicated for



*Arion ater* digestive gland and unlike NADPH-cytochrome c reductase no soluble activity is present(see sections 3.1.1.1. and .3.1.1.2.). In all four of the species this enzyme activity peaks at a density of between 1.12 and 1.15 g cm<sup>-3</sup> and has a broad distribution characteristic of endoplasmic reticulum. In three of the four species (*Arion ater*, *Arion lusitanicus* and *Helix aspersa*) mannitol oxidase is mostly found in a single fraction (density = 1.15 g cm<sup>-3</sup>) clearly separated from the mitochondria (marker succinate dehydrogenase). This provides some evidence for the presence of "mannosomes" in these species. In *Limax flavus* mannitol oxidase also peaks at a density of 1.15 g cm<sup>-3</sup> but the activity is spread-out over several fractions and resembles the distribution of aromatic alcohol oxidase. The results for *Limax flavus* are therefore rather more difficult to interpret. Given the similarity of the distributions of mannitol oxidase and aromatic alcohol oxidase it is possible that they reside in the same organelle although it is impossible to say whether this localisation is endoplasmic reticulum or "mannosome". However if "mannosomes" are present in this species they respond somewhat differently to homogenisation and cannot be readily distinguished from the endoplasmic reticulum using the centrifugation conditions employed.

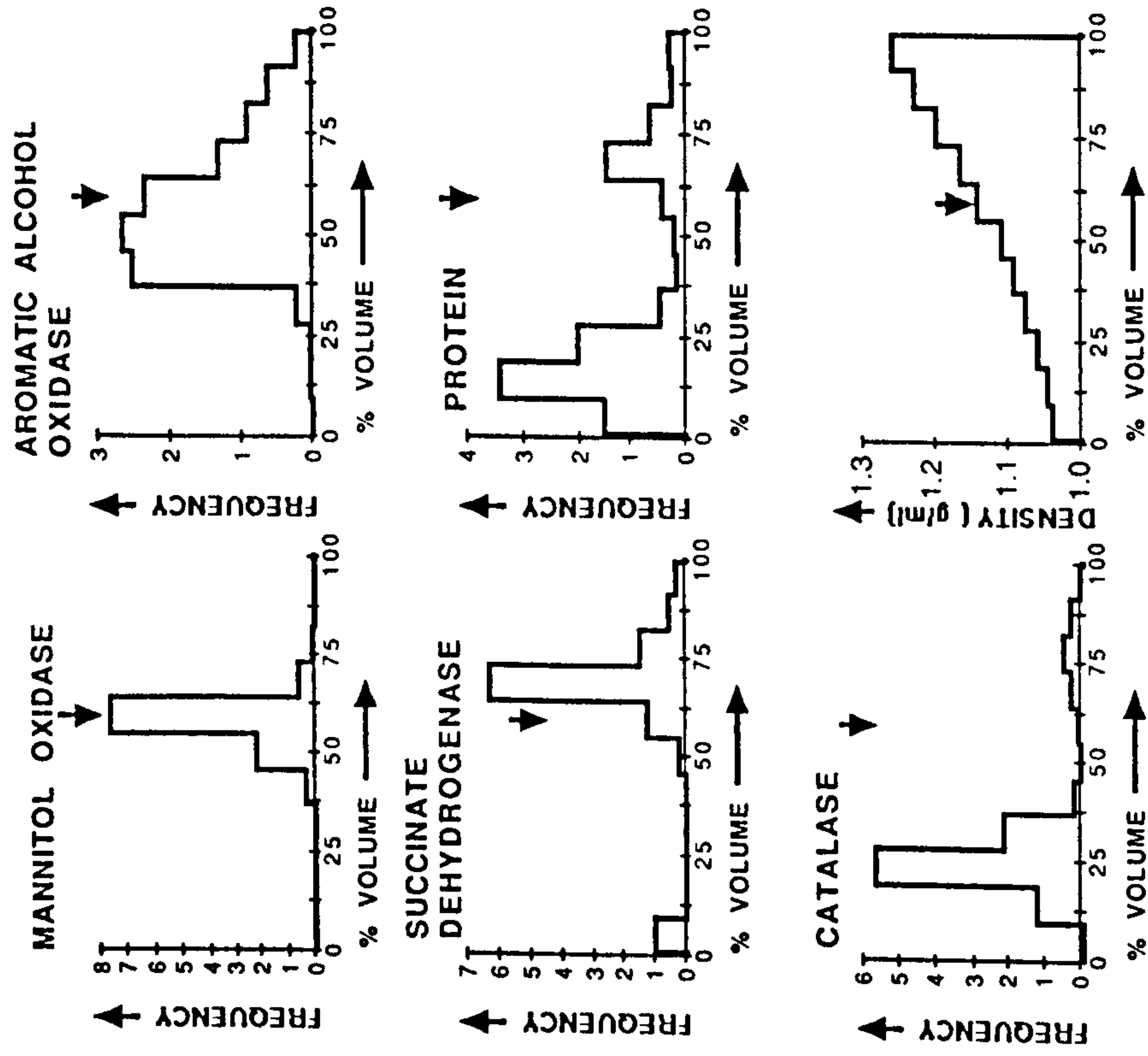


Figure 3.1.3.1.a) Isopycnic banding of *Arion ater* digestive gland P.N.S.. Centrifugation was at 45,000 r.p.m. for 2 hours using the Beckman Ti-50 vertical rotor. The arrow indicates the peak mannitol oxidase fraction.

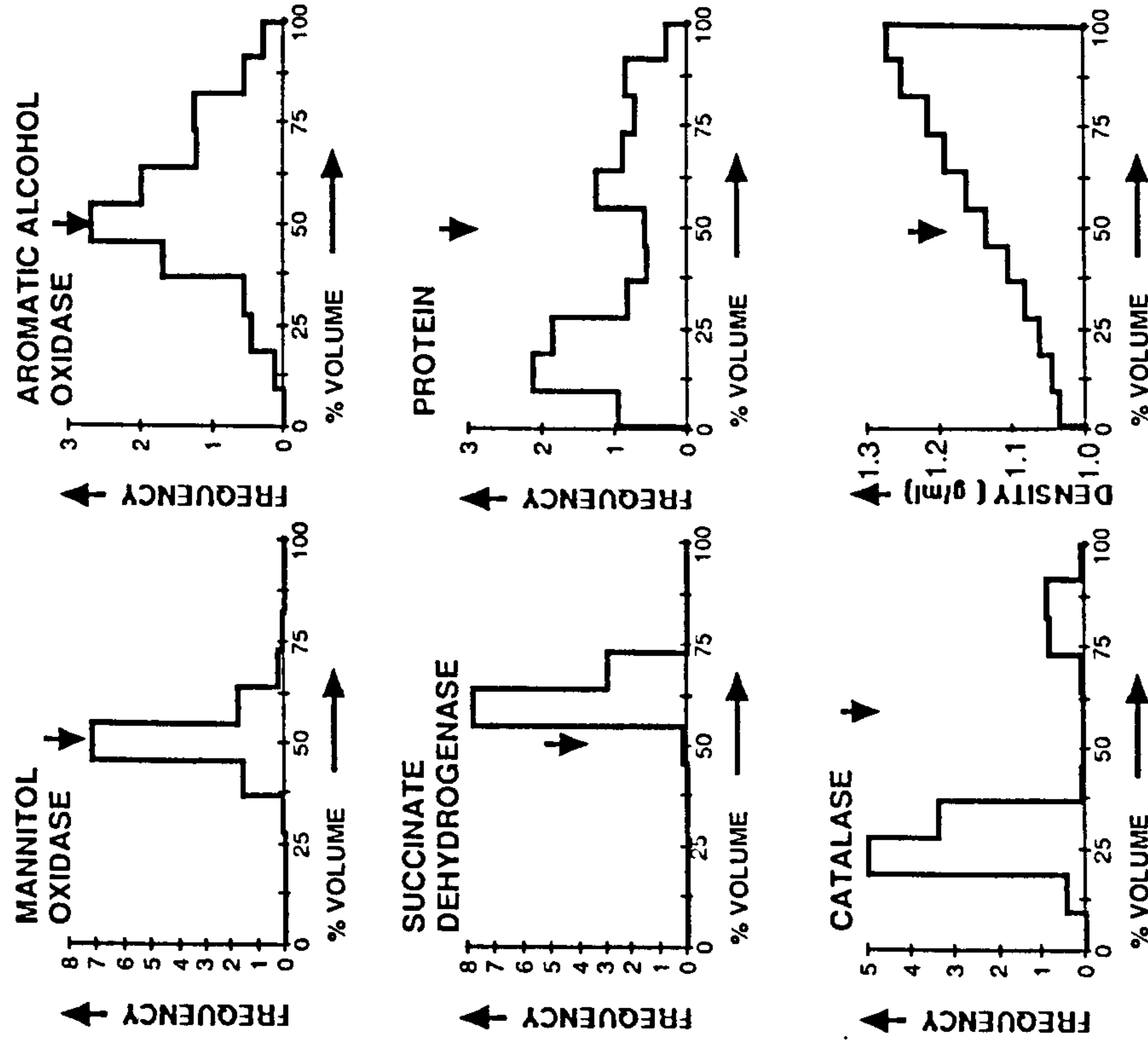


Figure 3.1.3.1.b) Isopycnic banding of *Arion lusitanicus* digestive gland P.N.S.. Centrifugation was at 45,000 r.p.m. for 2 hours using the Beckman Ti-50 vertical rotor. The arrow indicates the peak mannitol oxidase fraction.

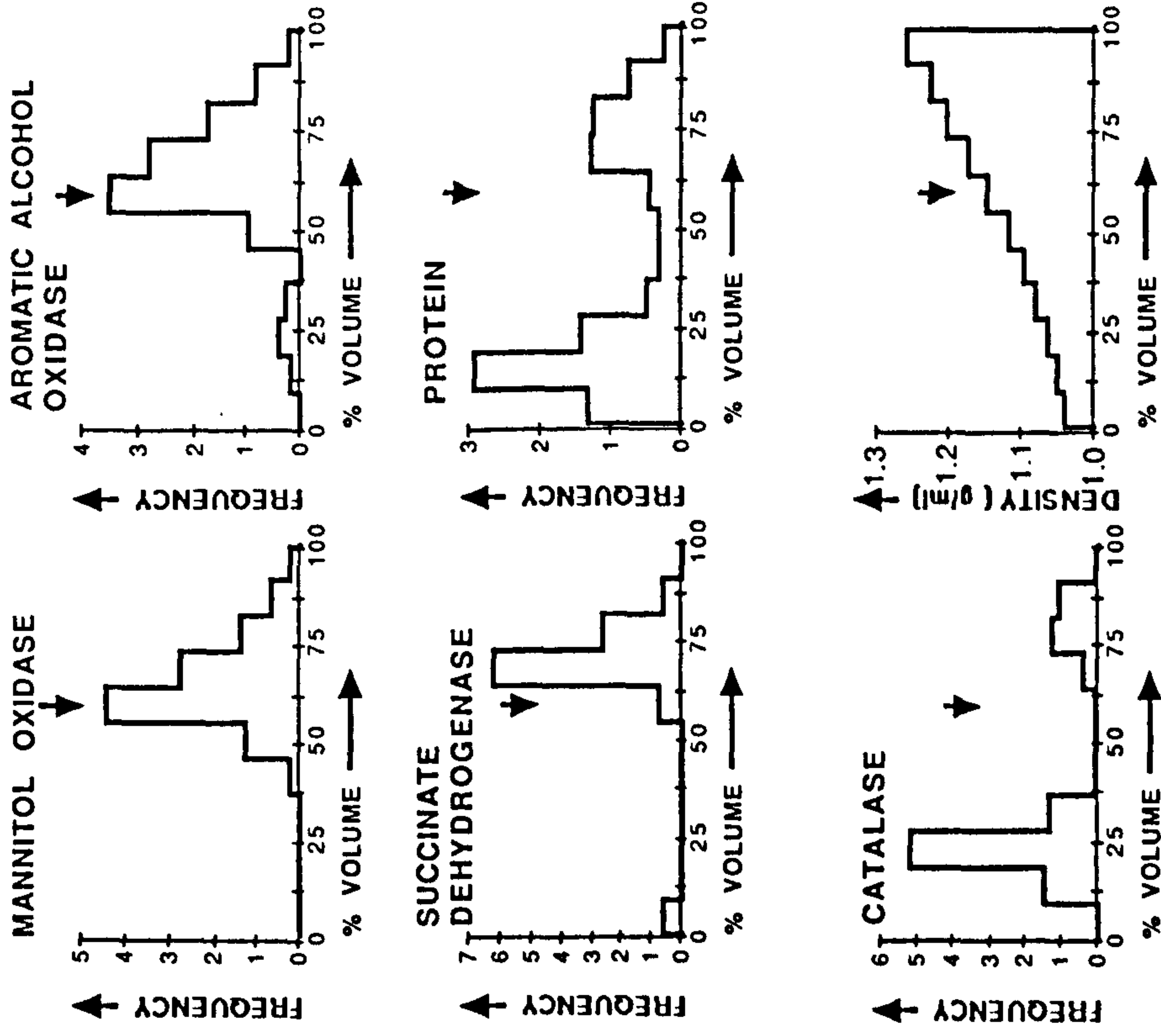


Figure 3.1.3.1.c) Isopycnic banding of *Limax flavus* digestive gland P.N.S.. Centrifugation was at 45,000 r.p.m. for 2 hours using the Beckman Ti-50 vertical rotor. The arrow indicates the peak mannitol oxidase fraction.

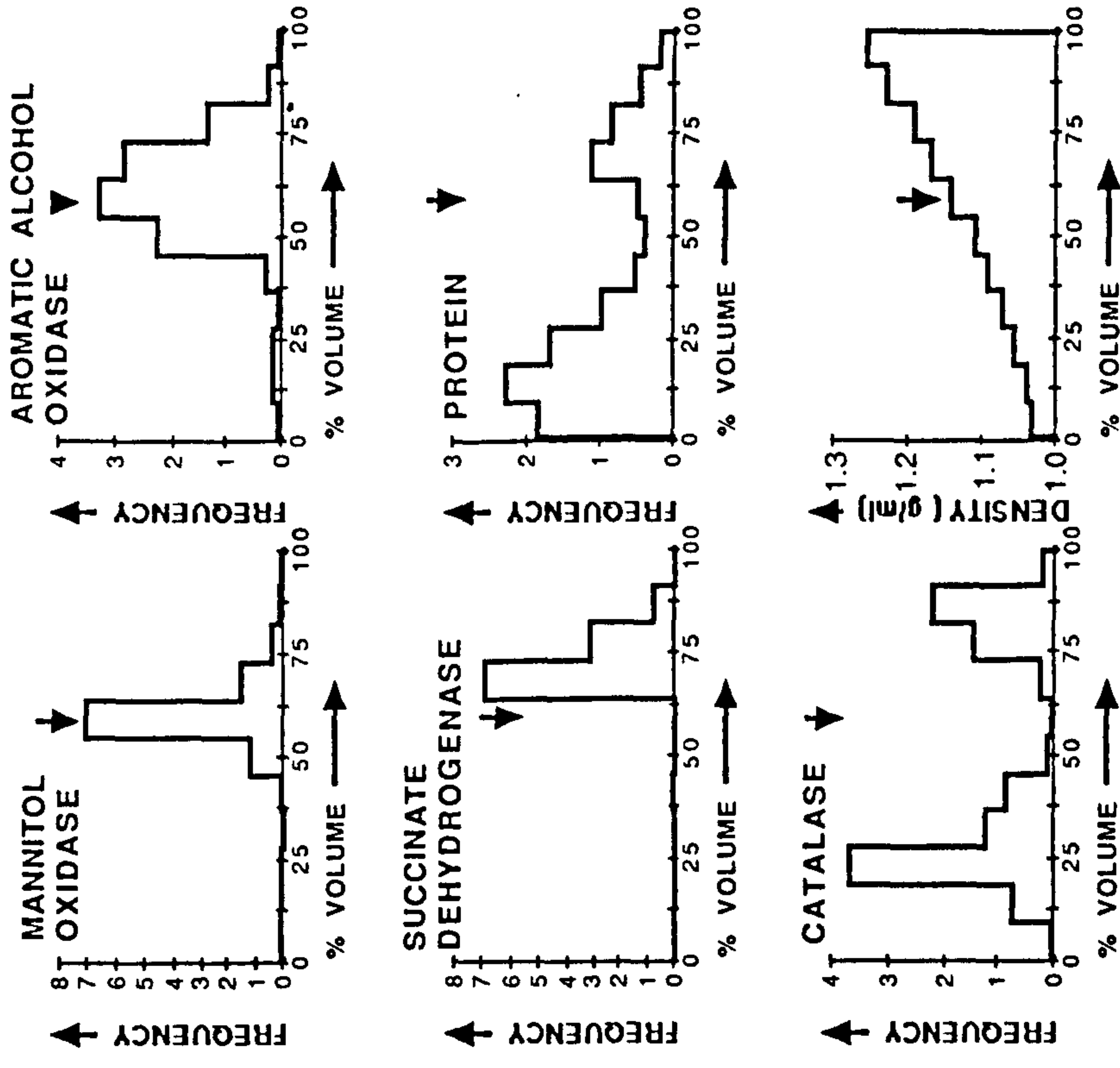


Figure 3.1.3.1.d) Isopycnic banding of *Helix aspersa* digestive gland P.N.S.. Centrifugation was at 45,000 r.p.m. for 2 hours using the Beckman Ti-50 vertical rotor. The arrow indicates the peak mannitol oxidase fraction.



### 3.1.3.2. Rate-dependent Banding

Digestive gland P.N.S. from the four species of gastropod mollusc described above was subjected to rate-dependent sedimentation in a shallow sucrose gradient using a 6 x 38ml M.S.E. swing-bucket rotor. Centrifugation was at 13,000 r.p.m. for 11 minutes (see section 2.2.2.6.). These experiments in conjunction with the density-dependent banding experiments (see previous section) were done so as to provide evidence for the presence of "mannosomes" in other species in addition to *Arion ater*.

The results of typical rate-dependent banding experiments are shown in figures 3.1.3.2.a) *Arion ater*, b) *Arion lusitanicus*, c) *Limax flavus* and d) *Helix aspersa*. In all cases the mitochondria (marker succinate dehydrogenase) are fast moving and reached the high-density cushion. Catalase activity is mostly soluble but the small proportion of particulate activity (peroxisomes) has tended to be fast-moving in all four species. With the exception of *Limax flavus* aromatic alcohol oxidase is associated with slower moving membranes (microsomes, e.g. endoplasmic reticulum). In *Limax flavus* aromatic alcohol oxidase is associated with slow-sedimenting membranes but some of the activity is clearly associated with faster-moving particles unlike in the other three species.

In *Arion ater*, *Arion lusitanicus* and *Helix aspersa* mannitol oxidase carrying structures have sedimented at an intermediate rate and have banded in the middle of the gradient. In these three species a good separation of mannitol oxidase from mitochondria (succinate dehydrogenase) and endoplasmic reticulum (aromatic alcohol oxidase) has been achieved. However in *Limax flavus* mannitol oxidase is associated with both slow- and fast-moving particles. In *Limax flavus* the distributions of aromatic alcohol oxidase and mannitol oxidase are rather similar in both rate and isopycnic

gradients. It is possible that these two activities may reside in the same organelle. It is likely that the two populations of mannitol oxidase revealed by rate-dependent sedimentation have been produced by homogenisation since the distribution of mannitol oxidase is fairly Gaussian in the isopycnic gradient. Whether this structure is endoplasmic reticulum or "mannosome" is however not known.

Therefore the results suggest that "mannosomes" are present in three of the four species investigated. The situation in *Limax flavus* is however unclear. If "mannosomes" are present in this species they respond somewhat differently to homogenisation. The subcellular localisation of aromatic alcohol oxidase is also unclear in this species as its distribution in both rate-dependent and isopycnic gradients is very similar to that of mannitol oxidase.

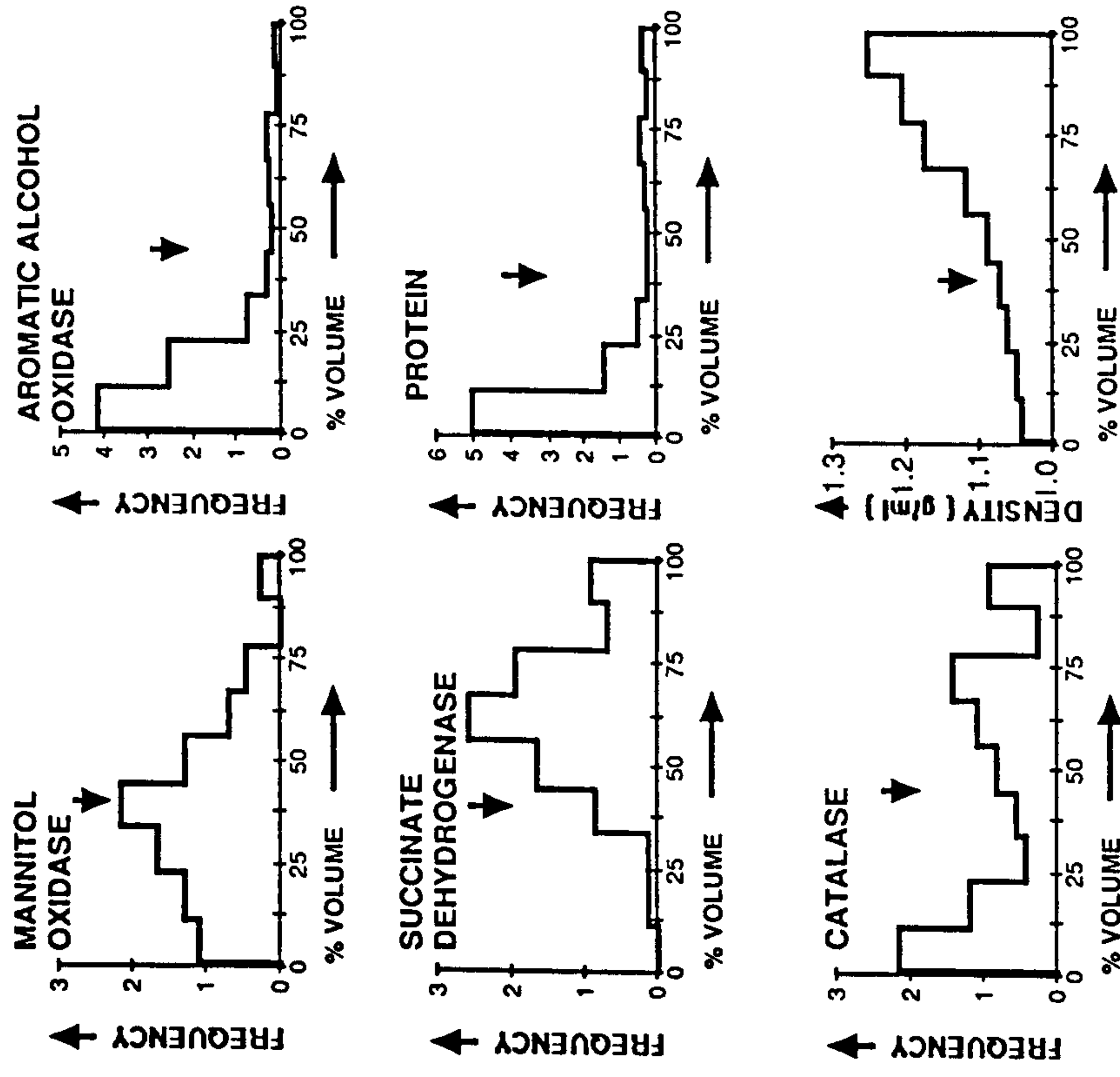


Figure 3.1.3.2.b) Rate-dependent banding of *Arion lusitanicus* digestive gland P.N.S.. Centrifugation was at 13,000 r.p.m. for 11 minutes using the M.S.E. 6 x 38ml swing-bucket rotor. The arrow indicates the peak mannitol oxidase fraction.

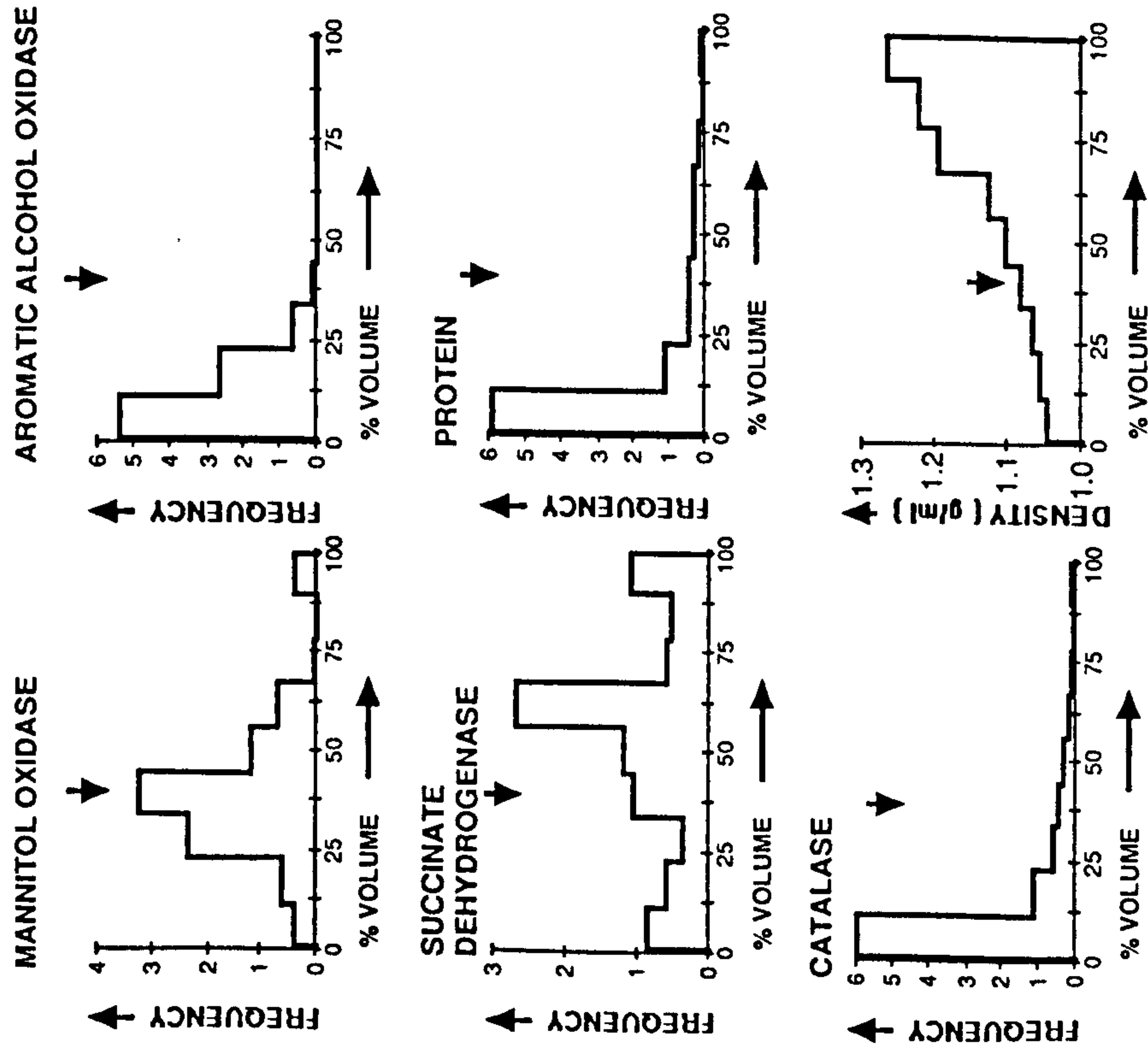


Figure 3.1.3.2.a) Rate-dependent banding of *Arion ater* digestive gland P.N.S.. Centrifugation was at 13,000 r.p.m. for 11 minutes using the M.S.E. 6 x 38ml swing-bucket rotor. The arrow indicates the peak mannitol oxidase fraction.



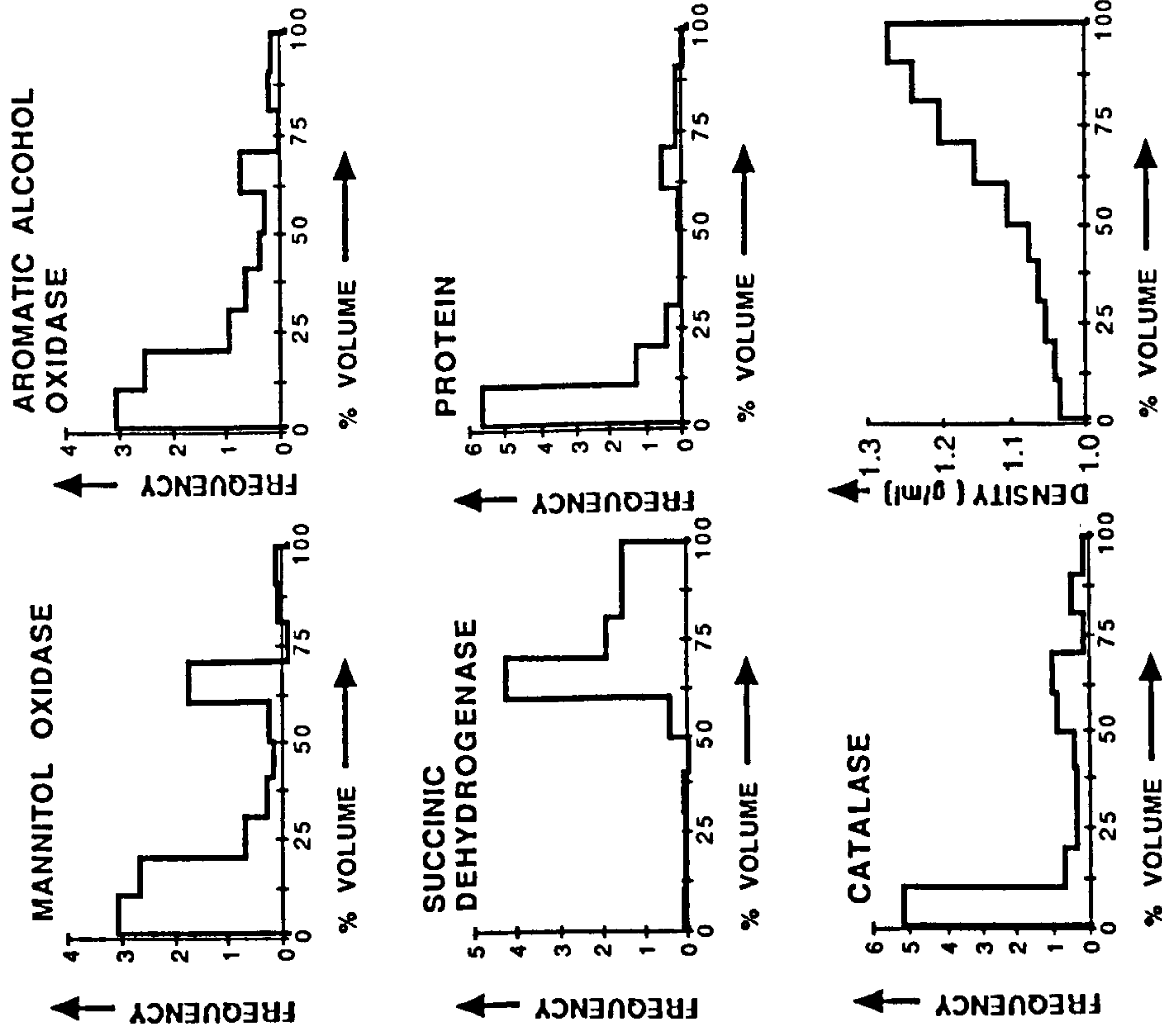


Figure 3.1.3.2.c) Rate-dependent banding of *Limax flavus* digestive gland P.N.S.. Centrifugation was at 13,000 r.p.m. for 11 minutes using the M.S.E. 6 x 38ml swing-bucket rotor. The arrow indicates the peak mannitol oxidase fraction.

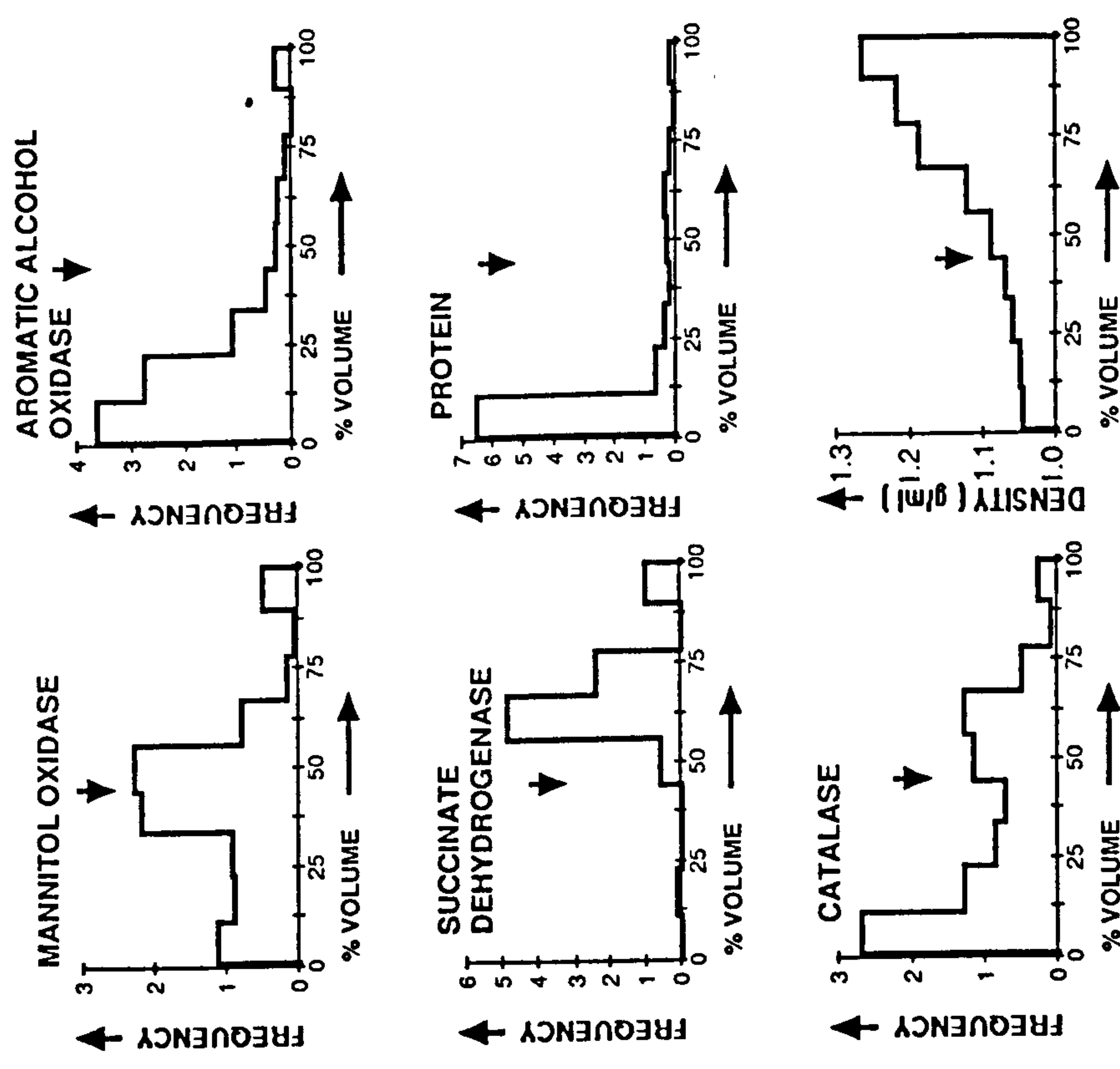
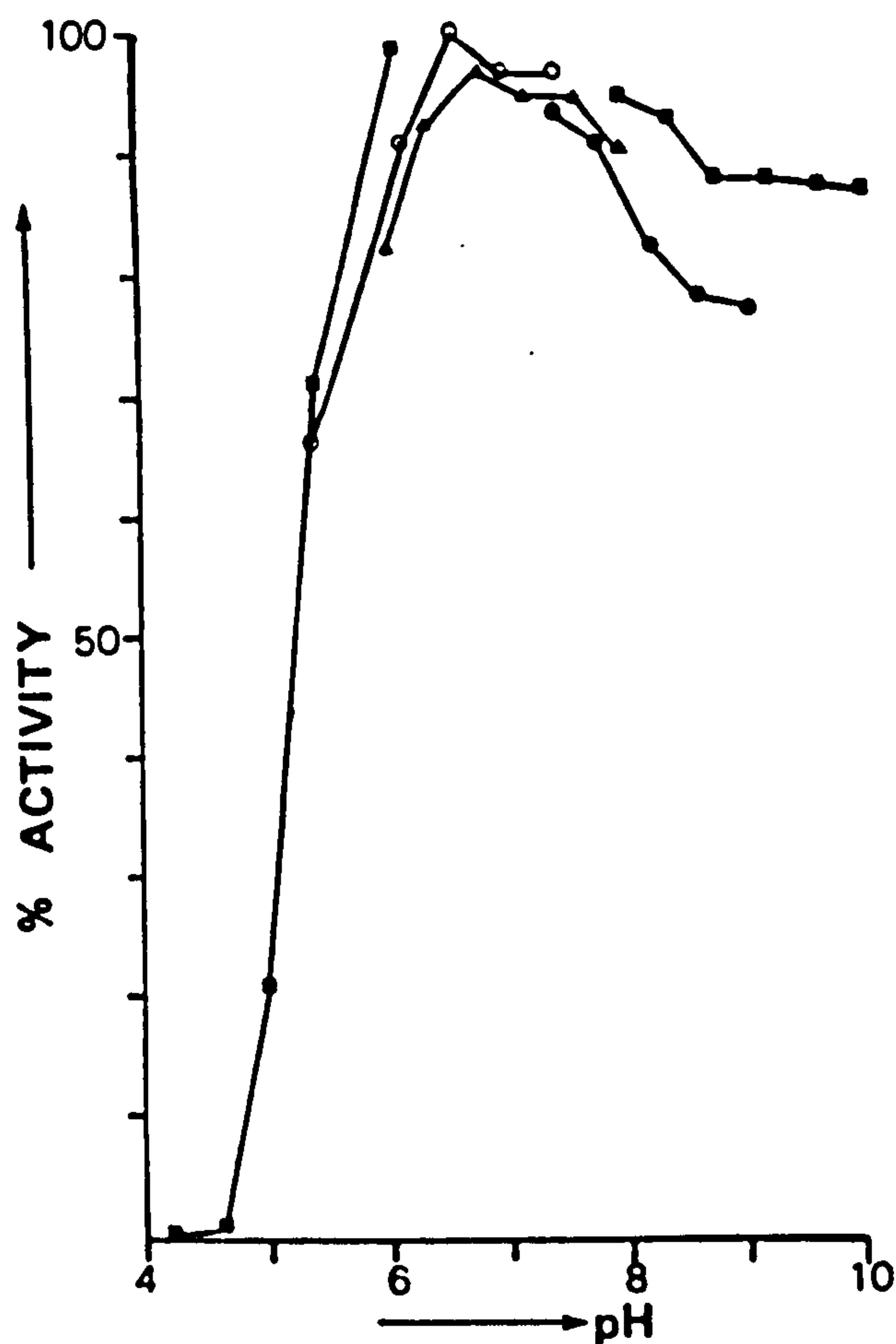


Figure 3.1.3.2.d) Rate-dependent banding of *Helix aspersa* digestive gland P.N.S.. Centrifugation was at 13,000 r.p.m. for 11 minutes using the M.S.E. 6 x 38ml swing-bucket rotor. The arrow indicates the peak mannitol oxidase fraction.



**Figure 3.2.1.1.a) Activity of aromatic alcohol oxidase with varying pH.**

The enzyme activity source was *Arion ater* digestive gland membrane fraction and activity was estimated by measuring the production of 2-naphthaldehyde at 250nm. The buffers used were sodium citrate (pH 4.0 -6.2), sodium cacodylate HCl (pH 5.0 - 7.4), potassium phosphate (pH 6.0 -8.0), tris-HCl (pH 7.4 - 9.0) and diethanolamine (pH 8.0 - 10.0) all at a concentration of 50mM. The absorbance of 2-naphthaldehyde was not affected by pH across the range used.

## 3.2. Characterisation of Catalytic Activities

### 3.2.1. Aromatic Alcohol Oxidase

#### 3.2.1.1. Aromatic Alcohol Oxidase Activity in *Arion ater*

Aromatic alcohol oxidase is a membrane-bound activity that is localised in the endoplasmic reticulum of the digestive gland of *Arion ater* (see section 3.1.1.1. and 3.1.1.2.). Activity could be quantitatively recovered from digestive gland homogenate by centrifugation at 100,000g for 2 hours. Because aromatic alcohol oxidase activity is membrane-bound it can be separated from contaminating aldehyde oxidase activity (which is soluble and cytosolic). The membrane-associated molluscan enzyme differs significantly from fungal aromatic alcohol oxidases which are extracellular soluble activities (see section 1.3.5.) The molluscan enzyme could not utilise the artificial electron - acceptor system of phenazine methosulphate (PMS) and 2,6-dichlorophenol-indophenol (DCPIP). The activity was also NAD(P)<sup>+</sup>-independent.

Using sedimented membranes derived from digestive gland homogenate as the enzyme source and following the appearance of 2-naphthaldehyde at 250nm the pH profile shown in figure 3.2.1.1.a) was obtained. The pH optimum for digestive gland particulate aromatic alcohol oxidase activity was 6.6 (cacodylate buffer). Substantial activity was found up to pH 10.0 but activity declined sharply below pH 5.0.

Particulate aromatic alcohol oxidase activity could be solubilised in a variety of detergents (table 3.2.1.1.a)). Ionic detergents (cationic, anionic and zwitterionic) all caused a significant loss of activity. Several non-ionic detergents proved to be effective solubilisers. Nonidet P40 was chosen as the most suitable detergent to use routinely because it gave a good yield (over 80% of the activity was



Detergent	Concentration (% w/v)	Solubility (%)	Inactivation (%)
<b>Non-ionic</b>			
Tween 20	0.2	3	-
Brij 35	0.25	26	-
Lubrol WX	0.25	45	-
Nonidet P40	0.2	81	-
Triton X100	0.1	87	<5
n-Octyl- $\beta$ -D-glucoside	1.4	87	-
<b>Cationic</b>			
Cetyltrimethyl-ammonium bromide	0.05	44	63
<b>Anionic</b>			
Sodium cholate	0.5	37	56
Sodium deoxycholate	0.2	49	64
<b>Zwitterionic</b>			
CHAPS	0.5	40	47

**Table 3.2.1.1.a) Solubility of aromatic alcohol oxidase derived from *Arion ater* digestive gland in a variety of detergents.**

Sedimented membranes were dispersed in detergent solution containing 10mM potassium phosphate buffer pH 7.4 and centrifuged at 100,000g for 2 hours. The percentage solubility was estimated by comparing the activity in the supernatant with that remaining in the pellet. The percentage inactivation was estimated by comparison with a zero-detergent control, the activity in the pellet of the control being defined as 100%. All detergent concentrations were above the critical micelle concentration for that detergent and a high molar ratio of detergent to protein was used.

solubilised), was inexpensive compared to n-octyl- $\beta$ -D-glucoside and had a lower uv absorbance compared to Triton X100.

It was found that an initial twenty-fold enrichment of aromatic alcohol oxidase could be achieved over the original homogenate by successive detergent treatments. Membrane fraction was first treated with 0.5% w/v Tween 20 and the resedimented activity solubilised with 1% w/v Nonidet P40 (method as described in section 2.3.1.1.c)). Further enrichment was possible by filtering the solubilised preparation through a DEAE-sepharose column equilibrated with 25mM phosphate buffer pH 7.4 containing 0.1% w/v Nonidet P40. The filtrate was enriched sixty-fold with respect to the original homogenate; however the yield was fairly poor. The results of a typical enrichment procedure are shown in table 3.2.1.1.b). This final step was usually omitted and for most experiments the Nonidet P40 solubilised activity was used. This was both readily obtainable in high yield and was not turbid (unlike membrane preparations).

Solubilised aromatic alcohol oxidase activity was tested with a variety of potential substrates. For most substrates the activity was estimated by following the production of hydrogen peroxide using the *l*-DCF-peroxidase linked assay (section 2.3.2.1.a)) in the presence of 15% v/v methanol. For coniferyl alcohol and 4-hydroxy-benzyl alcohol the activity was estimated by measuring the production of the corresponding aldehyde spectrophotometrically. The results of the substrate comparison experiments are shown in table 3.2.1.1c) and the structures of several potential substrates are shown in figure 3.2.1.1.b). Cinnamyl alcohol (*cis / trans* mixed isomers) was found to be the most favoured substrate under these conditions. 2-Naphthalene methanol was also an excellent substrate although 1-naphthalene methanol was not so favoured. Surprisingly the aromatic ethanol 2-naphthalene ethanol was not a substrate at all. 3-Methoxy- and 4-methoxy- benzyl alcohols were both good substrates but activity was not readily detectable with 2-methoxy-benzyl alcohol. Substitution at the 2-position on the ring (adjacent to the hydroxymethyl group) has a very marked effect on activity. This is also demonstrated by the observation that 1-naphthalene methanol is a much poorer substrate than 2-naphthalene methanol. Substitution at positions 3 and 4 have a

Fraction	Enrichment Factor (approx)	Yield
Homogenate	1	100%
Sedimented membranes	2.5	100%
Tween 20-washed membranes	10	97%
Nonidet P40 solubilised activity	20	78%
Post DEAE-sepharose filtrate	60	48%

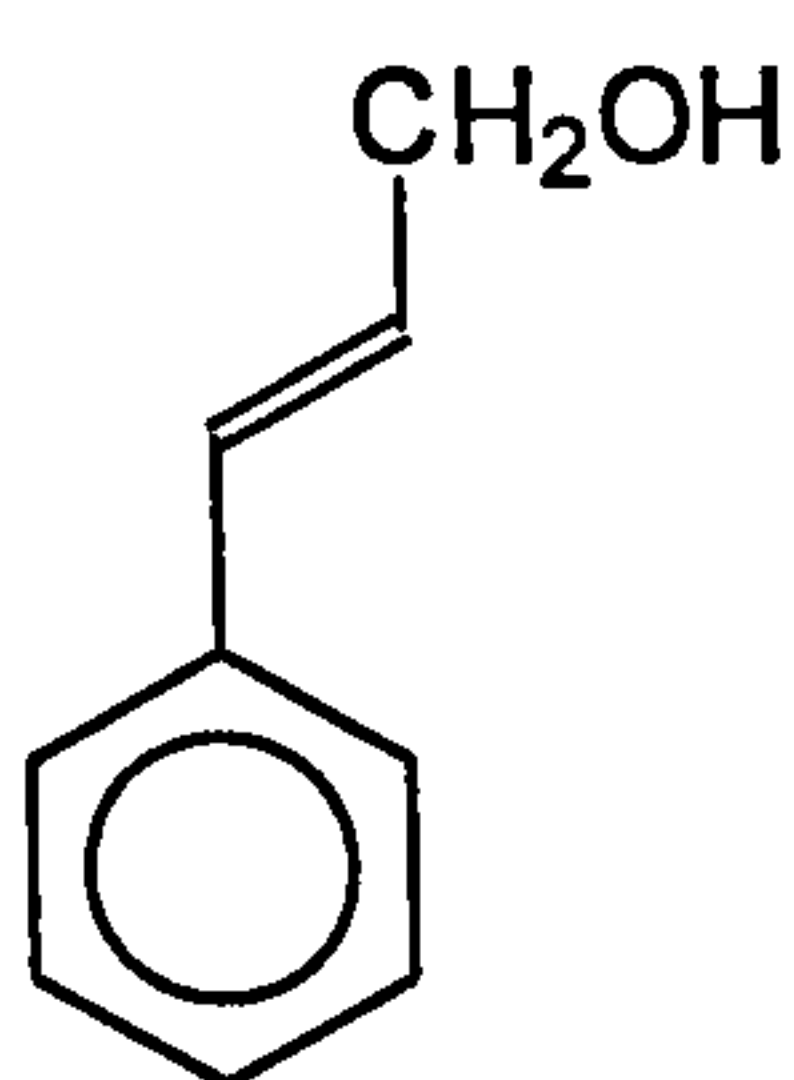
Table 3.2.1.1.b) Typical results obtained during the enrichment of aromatic alcohol oxidase from the digestive gland of *Arion ater*.  
The methods are as deccribed in the text. The enrichment factor is defined as the activity per milligram of protein in the fraction divided by the activity per milligram of protein in the tissue homogenate.



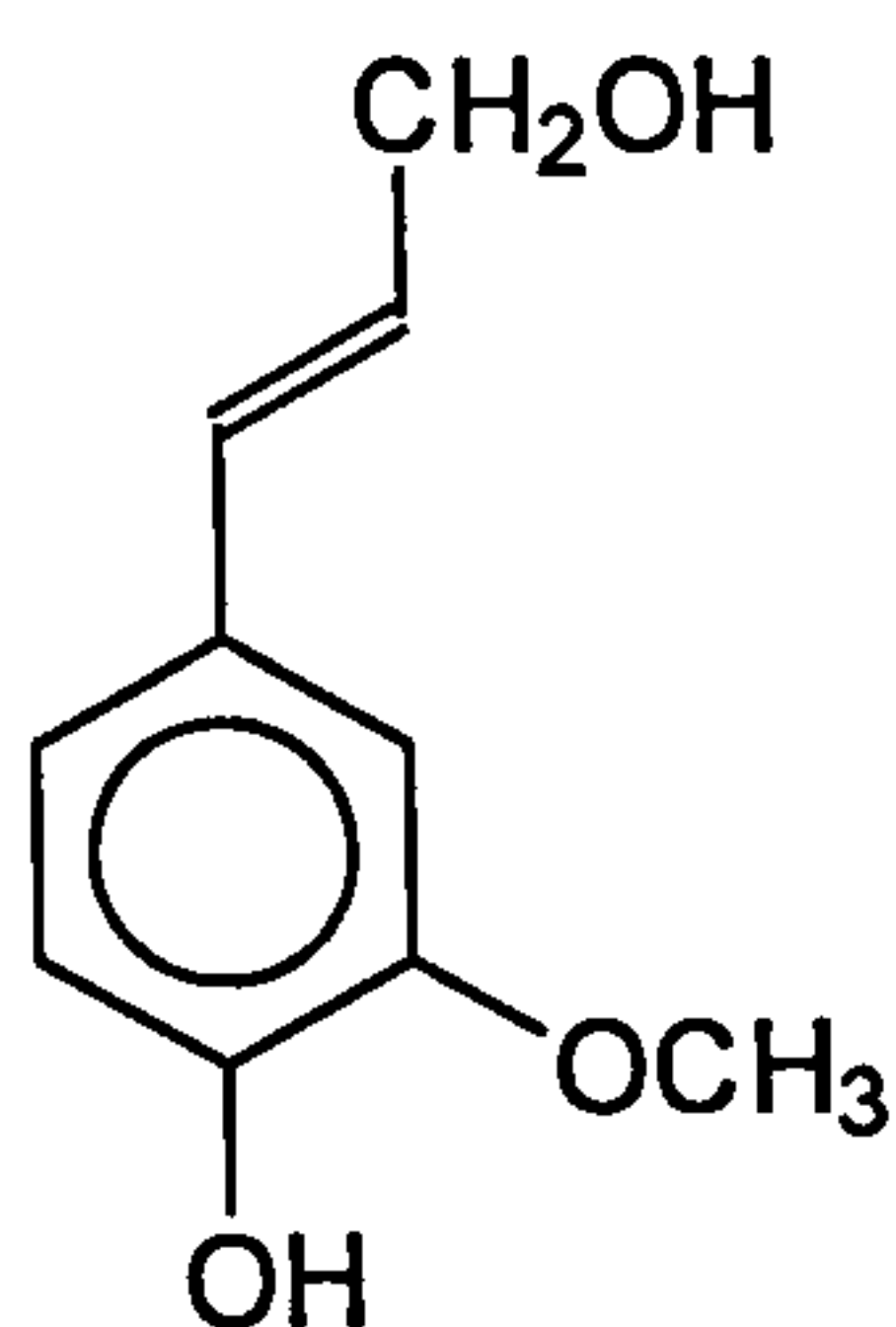
less significant effect as shown by the observation that 4-ethoxy-3-methoxy-benzyl alcohol and 3-ethoxy-4-methoxy-benzyl alcohol are equally favoured substrates. The high activity with 3-fluorobenzyl alcohol is rather unexpected although this may be due to the fluorine making other groups on the ring more reactive. Another surprising result is the measurable activity with cyclohexyl ethanol although cyclohexyl methanol is not a substrate. The lignin monomeric alcohol coniferyl alcohol (3-methoxy-4-hydroxy-cinnamyl alcohol) is a substrate although the activity with this substrate was estimated to only be about 14% of that with cinnamyl alcohol.

Using the solubilised enzyme activity and the *l*-DCF-peroxidase linked assay an apparent  $K_m$  value of  $35\mu\text{M}$  was obtained for 2-naphthalene methanol. By continuously monitoring of aldehyde production and using solubilised activity apparent  $K_m$  values of  $84\mu\text{M}$  for cinnamyl alcohol (290nm),  $91\mu\text{M}$  for 2-naphthalene methanol (250nm) and  $184\mu\text{M}$  for 4-methoxy-benzyl alcohol (290nm) were obtained at  $25^\circ\text{C}$  and pH7.4. All values were estimated by the Direct Linear Plot (Eisenthal and Cornish-Bowden 1974). The values obtained for the apparent  $K_m$  of 2-naphthalene-methanol by the direct and linked assays are somewhat different. This is probably due to the differing conditions in each of the assay methods. For example in the linked assay hydrogen peroxide was constantly removed by the peroxidase whereas no steps were taken to remove it in the direct assay. Also there was a relatively higher protein concentration in the linked assay because of the peroxidase present (at  $0.16\text{ mg cm}^{-3}$ ).

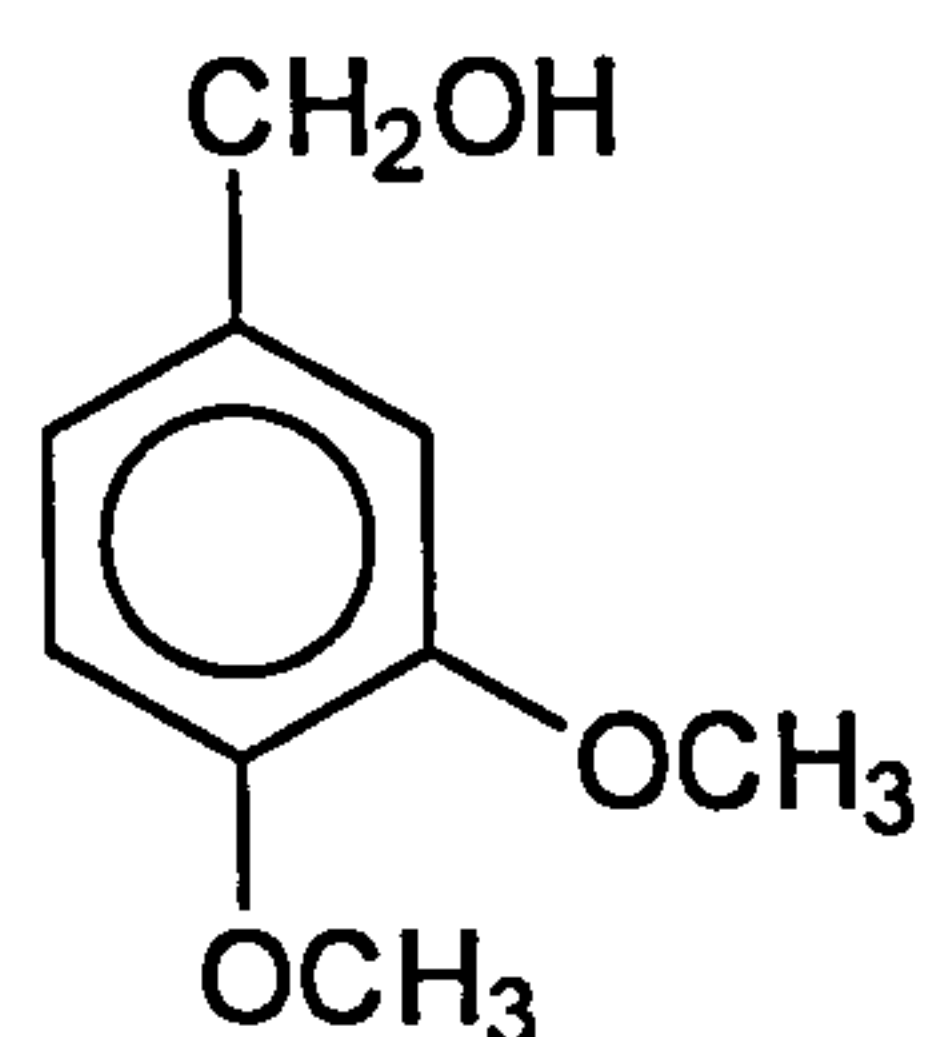
A stoichiometry for solubilised aromatic alcohol oxidase activity of 1.0 aldehyde to  $0.84 \pm 0.04$  ( $n=4$ )  $\text{H}_2\text{O}_2$  (mean  $\pm$  standard deviation) was found at  $25^\circ\text{C}$ . This was found by monitoring the appearance of cinnamaldehyde at 290nm and the production of hydrogen peroxide using the continuous TBHBA-AA-peroxidase linked assay (section 2.3.2.1.c)) separately. Conditions were kept as similar as possible in both assays; for example catalase was included in the assay measuring aldehyde production to remove hydrogen peroxide. The experiment was repeated using 2-naphthalene methanol as the substrate and monitoring the production of 2-naphthaldehyde at 250nm. A stoichiometry of 1.0 aldehyde to  $0.72 \pm 0.03$  ( $n=4$ )  $\text{H}_2\text{O}_2$



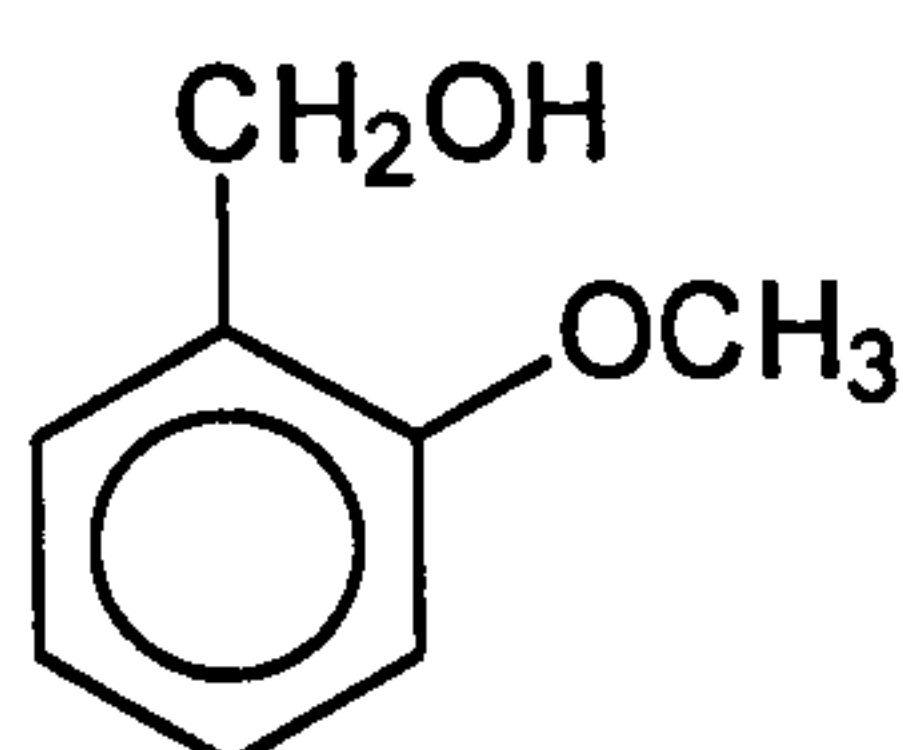
CINNAMYL  
ALCOHOL



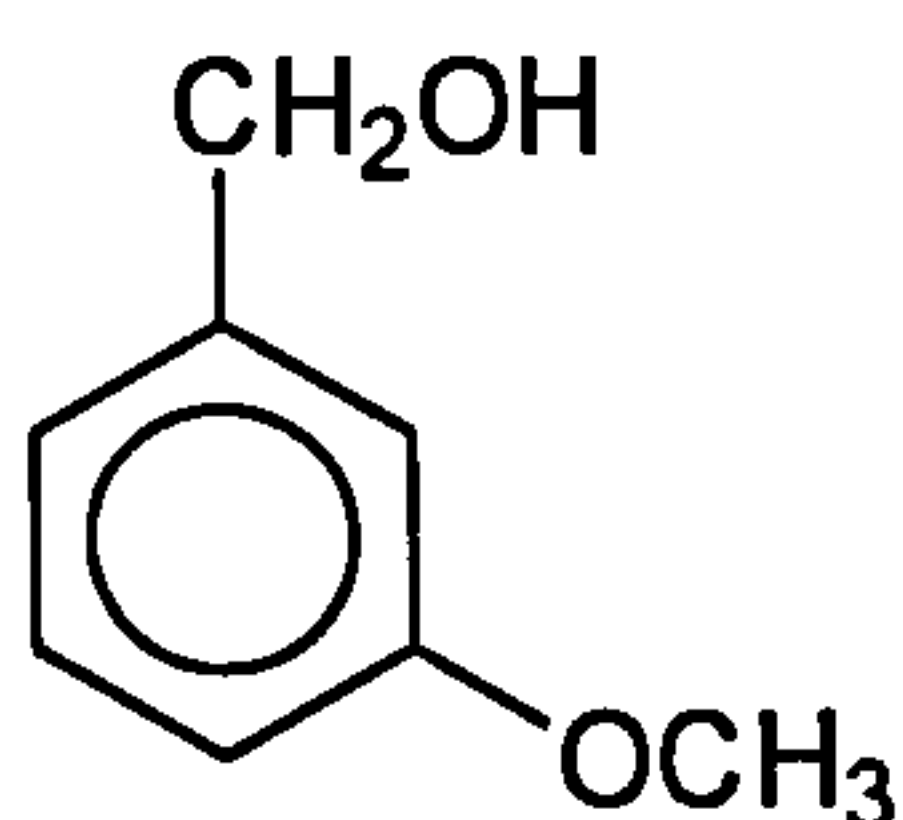
CONIFERYL  
ALCOHOL



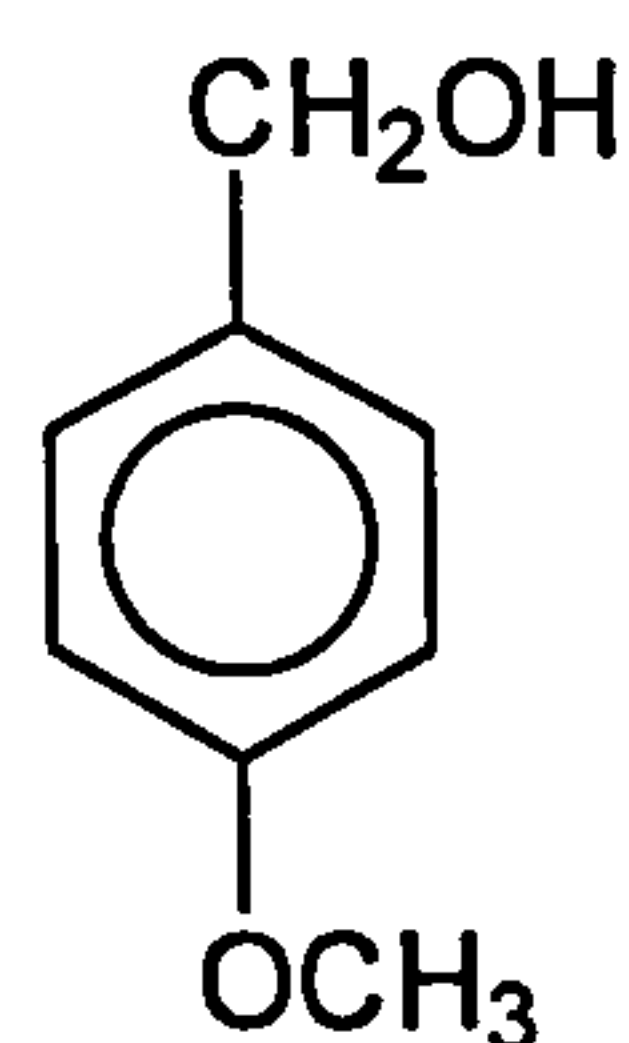
VERATRYL  
ALCOHOL



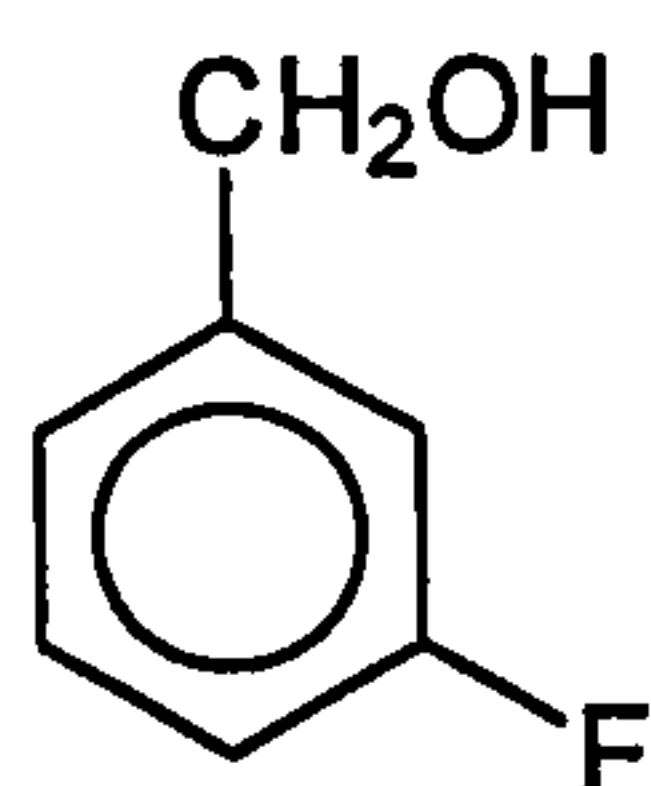
2-METHOXY-BENZYL  
ALCOHOL



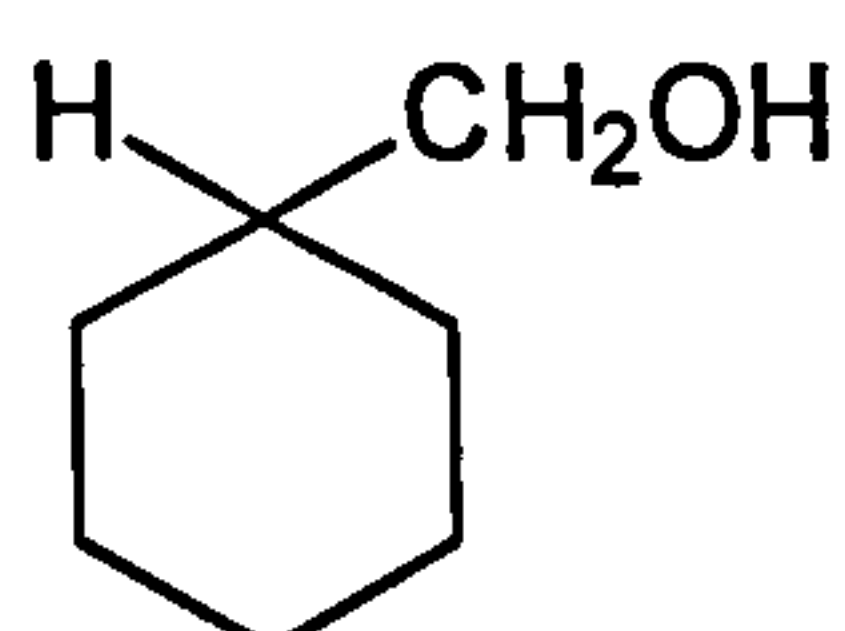
3-METHOXY-BENZYL  
ALCOHOL



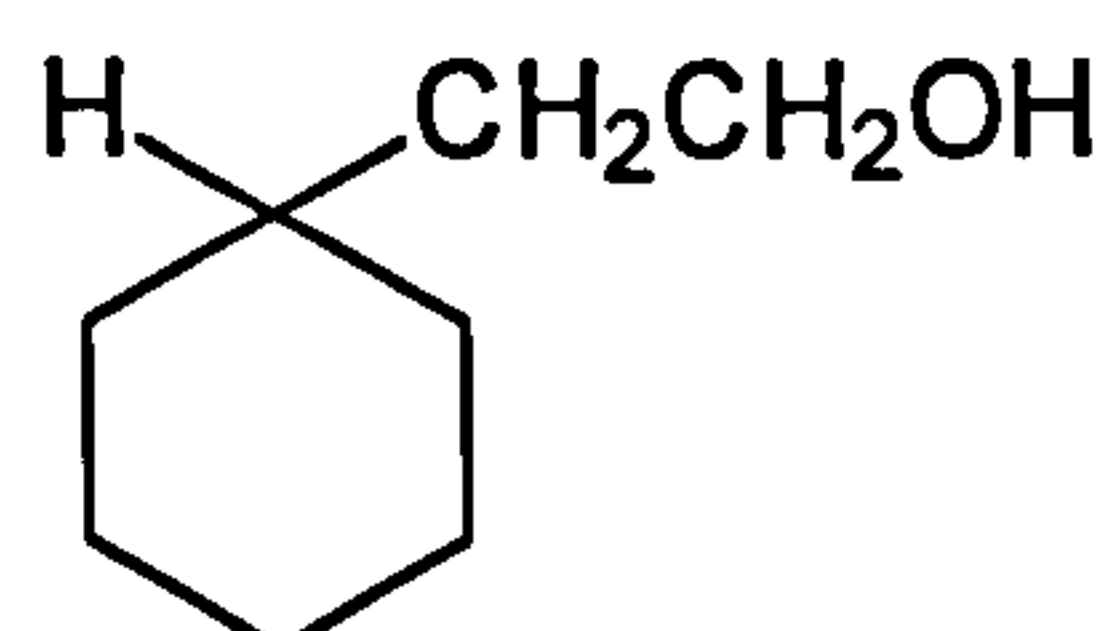
4-METHOXYBENZYL  
ALCOHOL



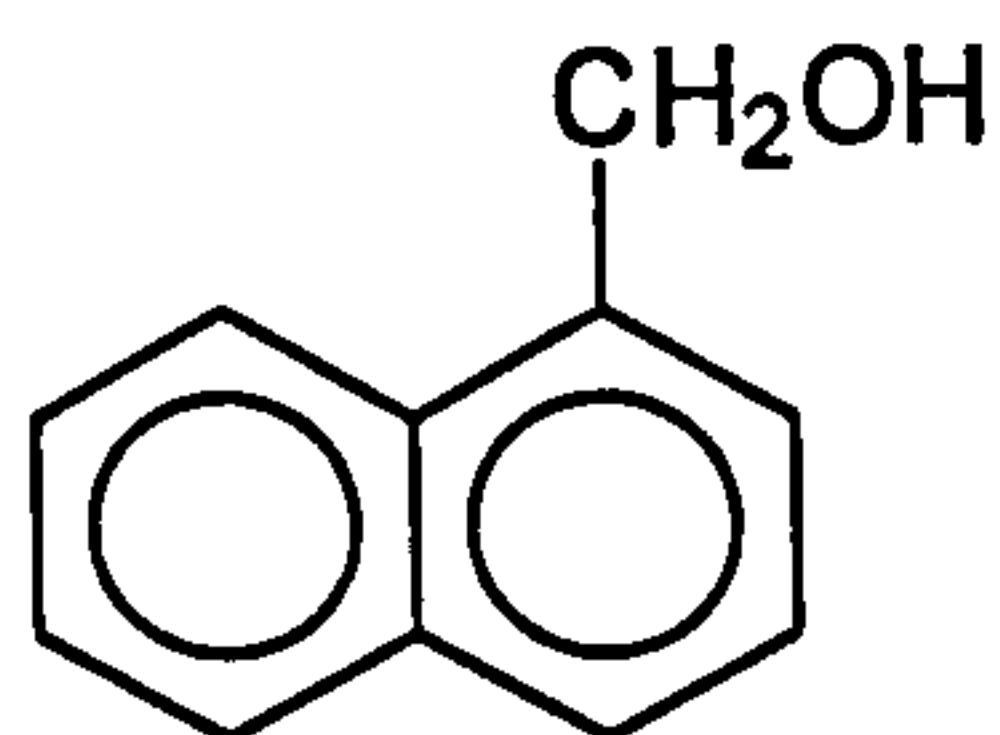
3-FLUORO-BENZYL  
ALCOHOL



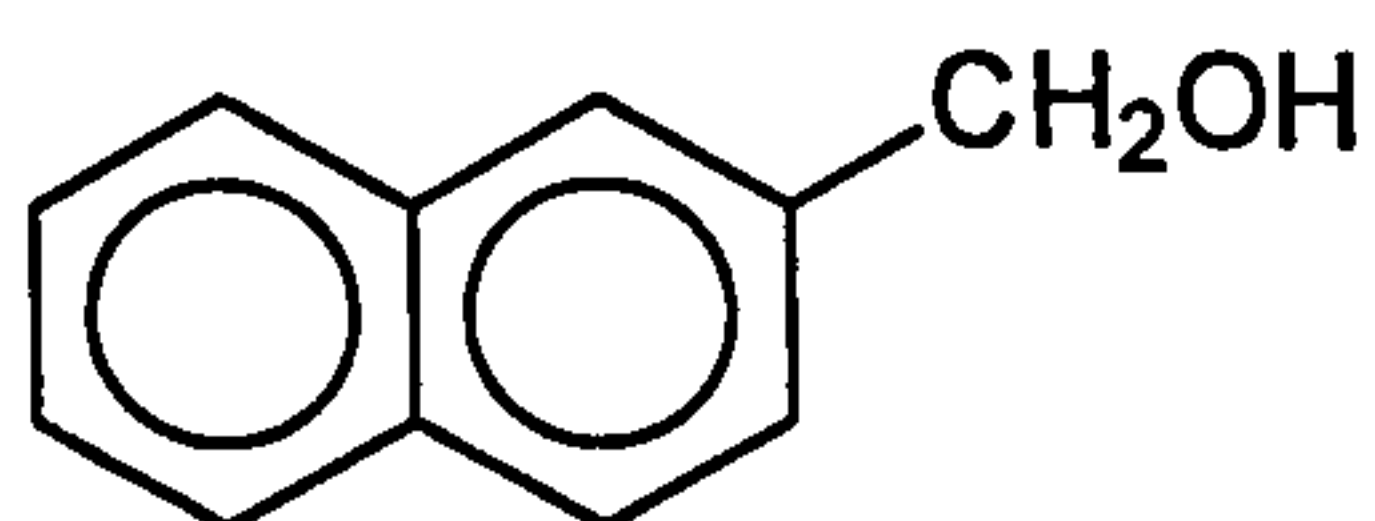
CYCLOHEXYL-  
METHANOL



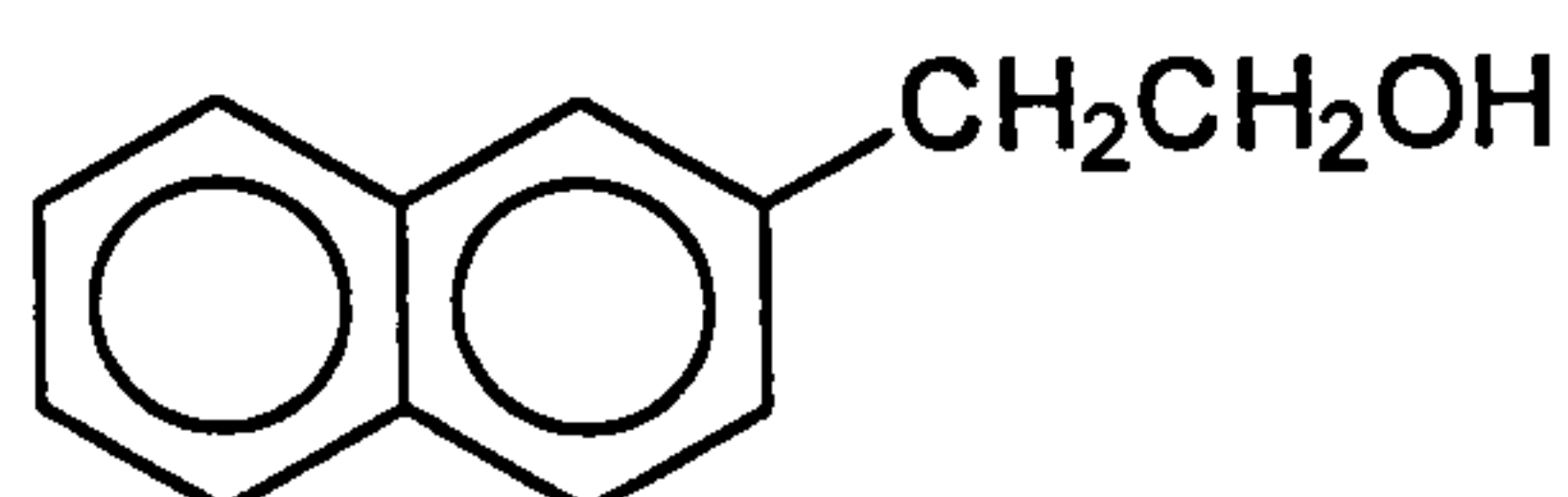
CYCLOHEXYL  
ETHANOL



1-NAPHTHALENE-  
METHANOL



2-NAPHTHALENE-  
METHANOL



2-NAPHTHALENE-  
ETHANOL

**Figure 3.2.1.1.b) - Structures of Potential Substrates  
for Aromatic Alcohol Oxidase**

Substrate	Activity (expressed as a percentage of that found with cinnamyl alcohol)
Cinnamyl alcohol	100
3-Fluorobenzyl alcohol	91
2-Naphthalene methanol	79
4-Methoxy-benzyl alcohol	62
3-Methoxy-benzyl alcohol	60
4-Ethoxy-3-methoxy-benzyl alcohol	52
3-Ethoxy-4-methoxy-benzyl alcohol	51
Veratryl alcohol	30
4-Amino-benzyl alcohol	29
1-Naphthalene methanol	27
Benzyl alcohol	26
3,5-Dimethoxy-benzyl alcohol	18
Cyclohexyl ethanol	14
Coniferyl alcohol <sup>a</sup>	14
3-Amino-benzyl alcohol	12
2-Methoxy-benzyl alcohol	<2
2-Naphthalene ethanol	<2
Cyclohexyl methanol	<2
4-Hydroxy-benzyl alcohol <sup>b</sup>	<2

**Table 3.2.1.1.c) Substrate preferences of solubilised aromatic alcohol oxidase from the digestive gland of *Arion ater*.**

Activity was estimated using the *l*-dichlorofluorescein-peroxidase linked assay to measure hydrogen peroxide production, substrates were at a concentration of 1.7mM and assays contained 15% v/v methanol (except where indicated).

<sup>a</sup> Activity with coniferyl alcohol (0.313mM) was estimated by measuring the production of coniferylaldehyde at 342nm ( $\epsilon = 56,800 \text{ M}^{-1} \text{ cm}^{-1}$ ). This was compared to activity with 2-naphthalene methanol (0.313mM) estimated by measuring the production of 2-naphthaldehyde at 250nm ( $\epsilon = 45,500 \text{ M}^{-1} \text{ cm}^{-1}$ ) which was arbitrarily defined as 79%. Assays contained 1% v/v methanol.

<sup>b</sup> Activity with 4-hydroxybenzyl alcohol (1mM) was estimated by measuring the production of 4-hydroxy-benzaldehyde at 300nm ( $\epsilon = 8,800 \text{ M}^{-1} \text{ cm}^{-1}$ ). This was compared to activity with cinnamyl alcohol (1mM) estimated by measuring the production of cinnamaldehyde at 290nm ( $\epsilon = 26,200 \text{ M}^{-1} \text{ cm}^{-1}$ ). Assays were in totally aqueous phase.



SUBSTRATE	SPECIES		
	<i>Arion ater</i>	<i>Limax flavus</i>	<i>Helix aspersa</i>
Cinnamyl alcohol	100%	100%	100%
Veratryl alcohol	18%	38%	54%
4-Methoxybenzyl alcohol	50%	55%	95%
3-Methoxybenzyl alcohol	60%	54%	83%
2-Methoxybenzyl alcohol	<5%	31%	69%
4-Aminobenzyl alcohol	18%	36%	116%
3-Aminobenzyl alcohol	7%	41%	99%
Crotyl alcohol	40%	50%	63%
Amyl alcohol	<2%	<2%	<2%

**Table 3.2.1.2. Substrate specificity of solubilised aromatic alcohol oxidase activity from the digestive gland of three species of gastropod mollusc.**

Activity was estimated by measuring hydrogen peroxide production using the *l*-DCF-peroxidase linked assay. Substrates were at a concentration of 1mM in totally aqueous phase. Activity with cinnamyl alcohol was arbitrarily defined as 100%.

(mean  $\pm$  standard deviation) was found. In both cases the ratio is less than 1.0:1.0 but this could be due to the unstable nature of hydrogen peroxide.

Solubilised activity was unsuitable for experiments using the oxygen electrode for several reasons. The relative insensitivity of the apparatus required the use of a more concentrated sample of aromatic alcohol oxidase. The detergent solution also tended to trap air-bubbles. Therefore a membrane preparation was used. Oxygen consumption and hydrogen peroxide production were measured simultaneously (see section 2.3.2.1.e)). A stoichiometry of 1.0 [O<sub>2</sub>] to  $0.75 \pm 0.01$  (n=4) H<sub>2</sub>O<sub>2</sub> (mean  $\pm$  standard deviation) was found at 22°C using cinnamyl alcohol as the substrate.

### 3.2.1.2. Comparison of Aromatic Alcohol Oxidase Activity in Three Species of Terrestrial Gastropod Mollusc

Membrane-bound aromatic alcohol oxidase activity was also found in the terrestrial gastropods *Helix aspersa* and *Limax flavus*. This activity was compared to the activity in *Arion ater*. Activity from all three species was solubilised in 1.0% w/v Nonidet P40, 10mM potassium phosphate buffer pH 7.4; the percentage solubilisation was comparable in all three species. Solubilised activity was used for all experiments. Potential substrates were tested at a concentration of 1mM and in totally aqueous phase at 25°C using the *l*-DCF-peroxidase linked assay to monitor hydrogen peroxide production. The results are shown in table 3.2.1.2.. Cinnamyl alcohol (*cis* /*trans* mixed isomers) was found to be an excellent substrate in all three species. There was considerable variation in the level of activity with amino-substituted benzyl alcohols. These were poor substrates for the *Arion* enzyme but moderate substrates for the *Limax* enzyme. Surprisingly the activity of the *Helix* enzyme with 3-amino- and 4-amino-benzyl alcohol was comparable to that found with cinnamyl alcohol with 4-amino-benzyl alcohol being the best substrate of those tested. No activity was

detectable for 2-methoxy-benzyl alcohol with the *Arion* enzyme although both the *Limax* and the *Helix* enzymes could utilise this substrate. Activity with aliphatic crotyl alcohol (but-2-ene-1-ol, *cis* / *trans* mixed isomers) was appreciable in all three species although activity with amyl alcohol (pentan-1-ol) was not readily detectable in any of the species. The observation that the gastropod enzymes are active with an aliphatic alcohol that has an allyl group adjacent to the hydroxy-methyl group implies that aromatic methanols are more likely to be substrates than aromatic ethanols. This is confirmed by the lack of activity with 2-naphthalene ethanol found with the *Arion* enzyme although 2-naphthalene methanol is an excellent substrate (see section 3.2.1.1.) The *Helix* enzyme appears to have the broadest substrate specificity of the three species studied. The *Arion* enzyme is the most limited in the range of substrates that can be utilised.

The results with the *Arion ater* enzyme for some substrates compared to cinnamyl alcohol in totally aqueous phase (substrates at 1mM) were found to be slightly different to those found in the presence of 15% w/v methanol (substrates at 1.7mM) (see section 3.2.1.1.). This could be due to the lower concentration used in totally aqueous phase not fully saturating the enzyme or because the methanol in some way affects the acceptability of some substrates to the enzyme.

By monitoring the production of cinnamaldehyde at 290nm apparent  $K_m$  values for cinnamyl alcohol (*cis* / *trans* mixed isomers) of 84 $\mu$ M (*Arion ater*), 29 $\mu$ M (*Limax flavus*) and 18 $\mu$ M (*Helix aspersa*) were found at 25°C. Values were estimated using the Direct Linear Plot (Eisenthal and Cornish-Bowden 1974).



Tissue (n=4)	mg tissue / g snail	Alcohol oxidase (specific activity) (nmol min <sup>-1</sup> mg protein <sup>-1</sup> )
Digestive gland	67.9 ± 9.5	0.87 ± 0.22
Kidney	11.2 ± 0.4	10.86 ± 3.5
Crop	5.4 ± 1.0	0.71 ± 0.42
Stomach + Intestine	19.1 ± 2.1	< 0.1
Reproductive duct	20.9 ± 1.6	< 0.1
Albumin Gland	100 ± 16.8	Not detected

**Table 3.2.1.3.a) Alcohol oxidase activity in tissues of *Achatina fulica*.**

Activity was estimated by measuring hydrogen peroxide production using the TBHBA-AA-peroxidase linked assay. The results are presented in the form of the mean ± the standard deviation (n=4).

### 3.2.1.3. Alcohol Oxidase Activity in *Achatina fulica*

Aromatic alcohol oxidase activity was found in the digestive gland of the tropical species *Achatina fulica* (giant african land snail) although this activity showed far more activity with aliphatic alcohols than was the case with the temperate species (see previous section). The large size of this gastropod afforded easier dissection and the tissue localisation of alcohol oxidase was investigated using cinnamyl alcohol (1mM) as the substrate. Activity was estimated by measuring hydrogen peroxide production using the TBHBA-AA-peroxidase linked assay (section 2.3.2.1.b)). The results are shown in table 3.2.1.3.a). The specific activity was highest in the kidney homogenate with the digestive gland having the next highest specific activity. However on a whole animal basis the digestive gland would contribute almost as much activity as the kidney because of its greater size.

As with *Arion ater* the particulate activity could be solubilised in detergents. Digestive gland membrane fraction previously treated with Tween 20 was extracted using 1.0% w/v Nonidet P40 in 10mM potassium phosphate buffer pH 7.4 (see section 2.3.1.1.c)). Greater than 98% of the activity could be solubilised in this way. The solubilised activity was tested with a variety of aliphatic and aromatic substrates in totally aqueous phase using the TBHBA-AA-peroxidase linked method to measure hydrogen peroxide generation. Aromatic substrates and crotyl alcohol were at a concentration of 10mM and saturated aliphatic substrates at a concentration of 100mM. The results are shown in table 3.2.1.3.b). The *Achatina* enzyme showed a number of marked differences to the enzymes of the species previously studied. Crotyl alcohol (but-2-ene-1-ol, *cis* / *trans* mixed isomers) was found to be the best substrate (at 10mM) of those tested rather than cinnamyl alcohol (*cis* / *trans* mixed isomers). Activity was also observed with the aromatic ethanol 2-phenyl ethanol. There was also measurable activity with primary aliphatic alcohols (ethanol, propan-1-ol, butan-1-ol and amyl alcohol (pentan-1-ol)) unlike the other gastropod activities studied (see previous section). Methanol and the secondary alcohol butan-2-ol were not utilised at all.

Substrate	Activity (expressed as percentage of that found with crotyl alcohol)
Crotyl alcohol	100%
Cinnamyl alcohol	74%
4-Methoxy-benzyl alcohol	64%
2-Phenyl ethanol	23%
Methanol	< 2%
Ethanol	21%
Propan-1-ol	33%
Butan-1-ol	40%
Butan-2-ol	< 2%
Amyl alcohol	33%

**Table 3.2.1.3.b) Substrate specificity of solubilised alcohol oxidase from *Achatina fulica*.**

Activity with crotyl alcohol was arbitrarily defined as 100%. Substrates were at a concentration of 10mM (aromatic alcohols and crotyl alcohol) or 100mM (saturated aliphatic alcohols) in totally aqueous phase. Activity was estimated by measuring hydrogen peroxide generation using the TBHBA-AA-peroxidase linked assay.

---

Substrate	Activity (expressed as a percentage of that found with amyl alcohol)
Amyl alcohol	100%
Hexan-1-ol	84%
Octan-1-ol	92%
Decan-1-ol	102%
Dodecan-1-ol	117%
Tetradecan-1-ol	85%
Hexadecan-1-ol	26%
Crotyl alcohol	72%
Cinnamyl alcohol	238%

**Table 3.2.1.3.c) Substrate specificity of solubilised alcohol oxidase from *Achatina fulica* - Activity towards long-chain alcohols.**

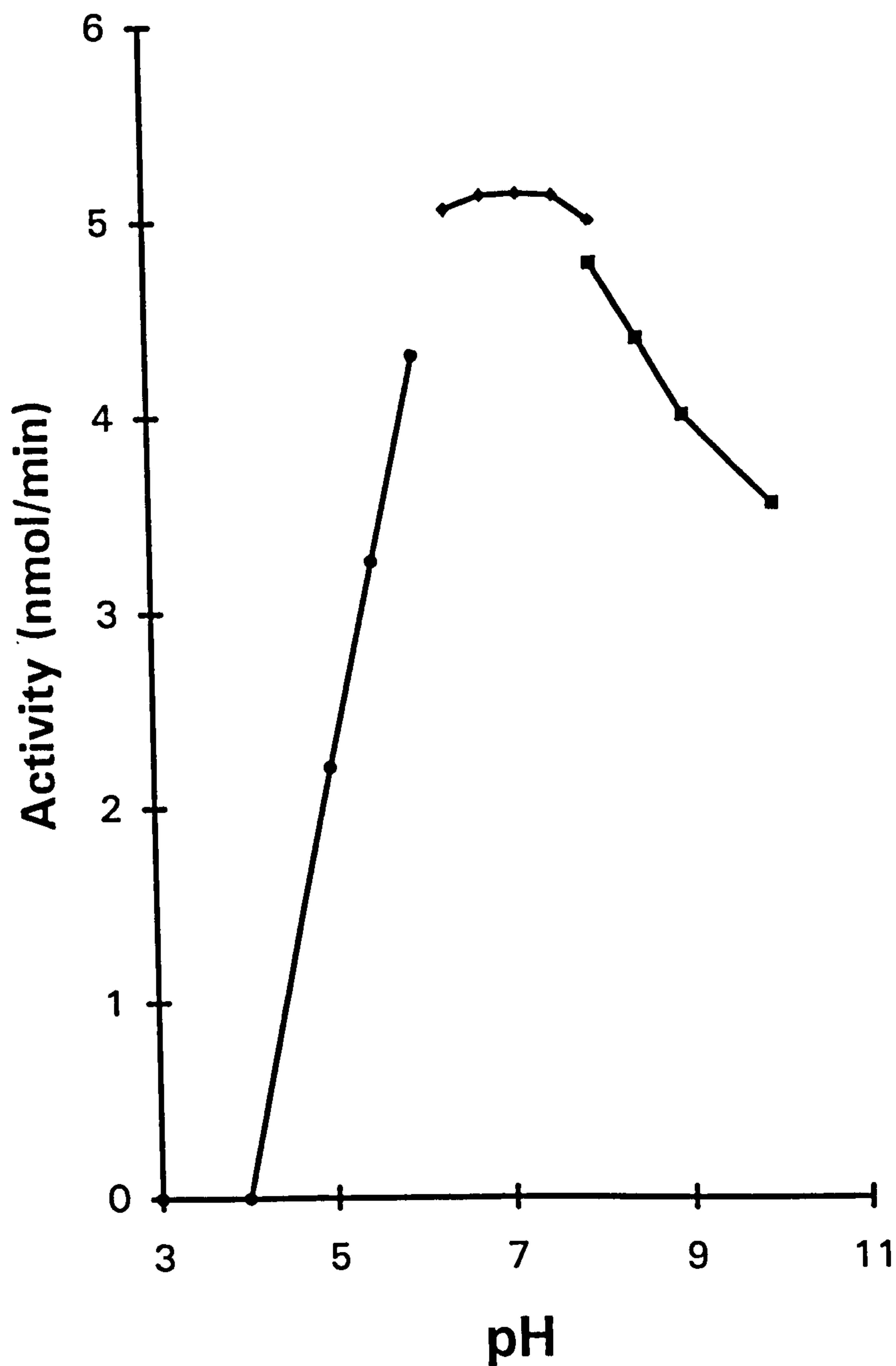
Activity with amyl alcohol was arbitrarily defined as 100%. All substrates were at a concentration of 250µM. Activity was estimated by measuring hydrogen peroxide generation using the TBHBA-AA-peroxidase linked assay. All assays contained 10% v/v methanol.



Apparent  $K_m$  values using the solubilised activity and the TBHBA-AA-peroxidase linked assay of 252  $\mu$ M for crotyl alcohol (*cis* / *trans* mixed isomers) and 146  $\mu$ M for cinnamyl alcohol (*cis* / *trans* mixed isomers) were found at 30°C. The apparent  $K_m$  for amyl alcohol was found to be too low to be reliably estimated using the TBHBA-AA-peroxidase linked assay so the more sensitive *l*-DCF-peroxidase linked assay for monitoring hydrogen peroxide production was employed. An apparent  $K_m$  value of 41  $\mu$ M was found at 30°C. All values were estimated using the Direct Linear Plot (Eisenthal and Cornish-Bowden 1974).

Activity with longer chain aliphatic alcohols (fatty alcohols) was also investigated using the TBHBA-AA-peroxidase linked assay. Substrates were tested at a concentration of 250  $\mu$ M and 10% v/v methanol was included in the assays to aid the solubility of the substrates. Activities were compared with that of both amyl alcohol and cinnamyl alcohol using the same conditions. The results are shown in table 3.2.1.3.c). Considerable activity was found up to at least C<sub>16</sub> (hexadecanol). A control assay lacking substrate but including 10% v/v methanol was included.

In order to confirm that activity with aliphatic and aromatic substrates was due to the same enzyme amyl alcohol and cinnamyl alcohol were supplied simultaneously in the same assay (TBHBA-AA-peroxidase linked method). The result when both substrates were supplied was compared to the result when the substrates were supplied separately. The activities were not additive when both substrates were supplied therefore activity with aliphatic and aromatic alcohols is probably due to the same enzyme. Activity with decanol and dodecanol was investigated in totally aqueous phase using particulate and solubilised enzyme preparations (for method see section 2.3.2.1.b)). With both of these virtually insoluble substrates the activity was at least 98% of that with amyl alcohol in totally aqueous phase using both particulate and solubilised enzyme activity. No activity was observed using methanol as the substrate.



**Figure 3.2.1.3. Activity of solubilised alcohol oxidase from *Achatina fulica* with varying pH.**

Cinnamyl alcohol (1mM) was used as the substrate and activity was estimated by measuring the appearance of cinnamaldehyde at 290nm. The buffers used were potassium acetate (100mM, pH 3.0-5.5), potassium phosphate (100mM, pH 6.0-8.0) and Glycine-NaOH (66mM, pH 8.0-10.0). The absorbance of cinnamaldehyde was unaffected across the pH range used.

By measuring cinnamaldehyde production at 290nm the activity with varying pH was investigated (figure 3.2.1.3.). A broad pH optimum of 6.4 - 8.0 was found for the solubilised enzyme with activity falling off dramatically below pH 5.0. Activity was found up to at least pH 10.0 (approximately 69% of the activity at optimum pH).



SUBSTRATE	SPECIES			
	<i>Arion ater</i>	<i>Limax flavus</i>	<i>Helix aspersa</i>	<i>Mus musculus</i>
Benzaldehyde	100%	100%	100%	100%
Veratraldehyde	65%	36%	48%	76%
3-Methoxybenzaldehyde	97%	70%	82%	77%
Cinnamaldehyde	54%	78%	60%	136%
3-Hydroxybenzaldehyde	91%	88%	93%	<5%
Salicylaldehyde	<3%	<3%	59%	88%
Hypoxanthine	0	0	0	0

**Table 3.2.2.1. Substrate specificities of aldehyde oxidase from the digestive gland of three species of gastropod mollusc and mouse liver.**

Activity is expressed as a percentage of that found with benzaldehyde which is arbitrarily defined as 100%. Substrates were at a concentration of 50µM (aldehydes) or 0.7mM (hypoxanthine). Activity was estimated by measuring hydrogen peroxide generation using the *l*-DCF-peroxidase linked assay.

### 3.2.2. Aldehyde Oxidase

#### 3.2.2.1. Comparison of Aldehyde Oxidase Activities in Three Species of Gastropod Mollusc and in Mouse Liver

Aldehyde oxidase activity was studied because of its possible role in the further oxidation of the products of aromatic alcohol oxidase. Aldehyde oxidase activities from the digestive gland homogenate 100,000g x 2 hours supernatants of three species of gastropod mollusc (*Arion ater*, *Limax flavus* and *Helix aspersa*) were compared to the aldehyde oxidase activity obtained from the 100,000g x 2 hours supernatant of mouse liver. A variety of potential substrates were tested with each of the four species; the results are shown in table 3.2.2.1.. Activity was estimated by measuring the aldehyde-dependent hydrogen peroxide production using the *l*-DCF-peroxidase linked assay (see section 2.3.2.2.). Substrates were at a concentration of 50 $\mu$ M (aldehydes) or 0.7mM (hypoxanthine) in totally aqueous phase. Benzaldehyde, veratraldehyde, cinnamaldehyde and 3-methoxy-benzaldehyde were found to be good substrates for the enzyme in all four species. Surprisingly the mammalian enzyme was not active with 3-hydroxy-benzaldehyde which was also found to be an inhibitor of this activity. Salicylaldehyde (2-hydroxy-benzaldehyde) was utilised by the mouse and *Helix* enzymes but was an inhibitor of the, *Limax* and *Arion* enzyme activities. Only the *Helix* enzyme could utilise both of these hydroxy-substituted substrates. No activity could be detected with hypoxanthine (0.7mM) with any of the enzyme sources indicating that the observed activities were due to aldehyde oxidase and not xanthine oxidase.

Apparent  $K_m$  values for benzaldehyde of 1.9 $\mu$ M (*Arion*), 11.9 $\mu$ M (*Limax*), 5.5 $\mu$ M (*Helix*) and 5.1 $\mu$ M (mouse) were found at 15°C (molluscs) or 30°C (mouse). Values were estimated by the Direct Linear Plot (Eisenthal and Cornish-Bowden 1974). Experiments

Figure 3.2.2.1.a)  $s/v$  against  $[i]$

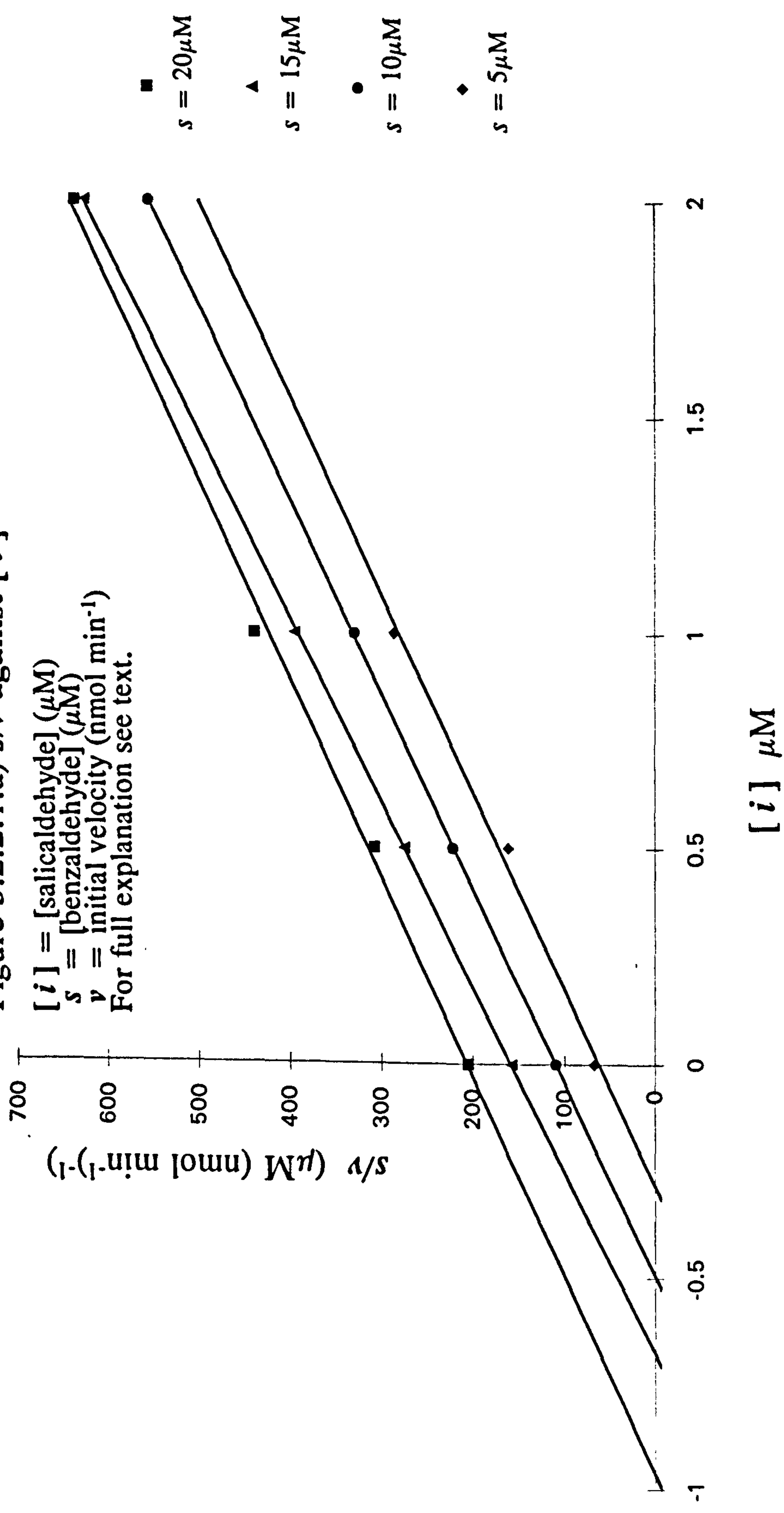
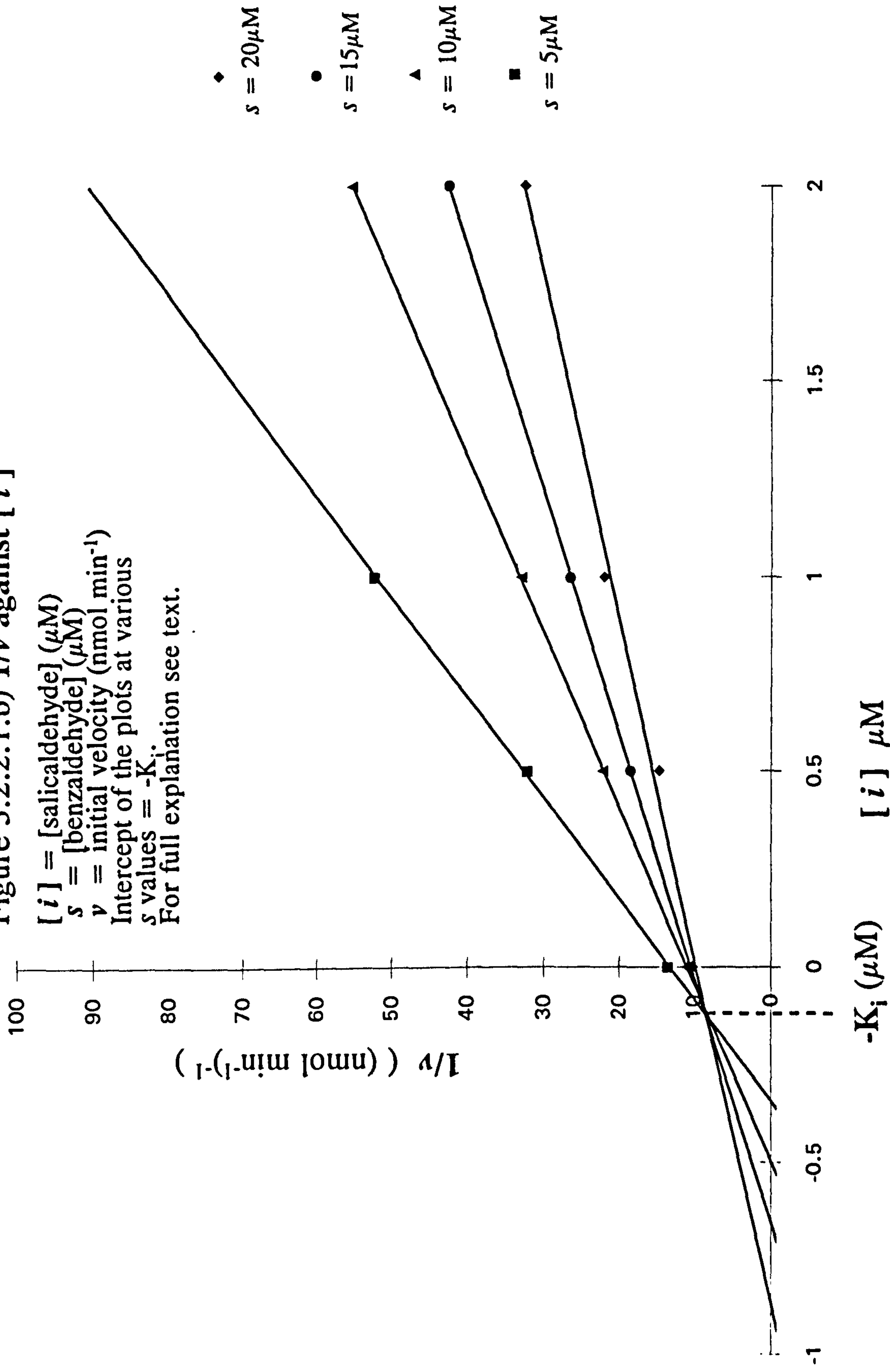
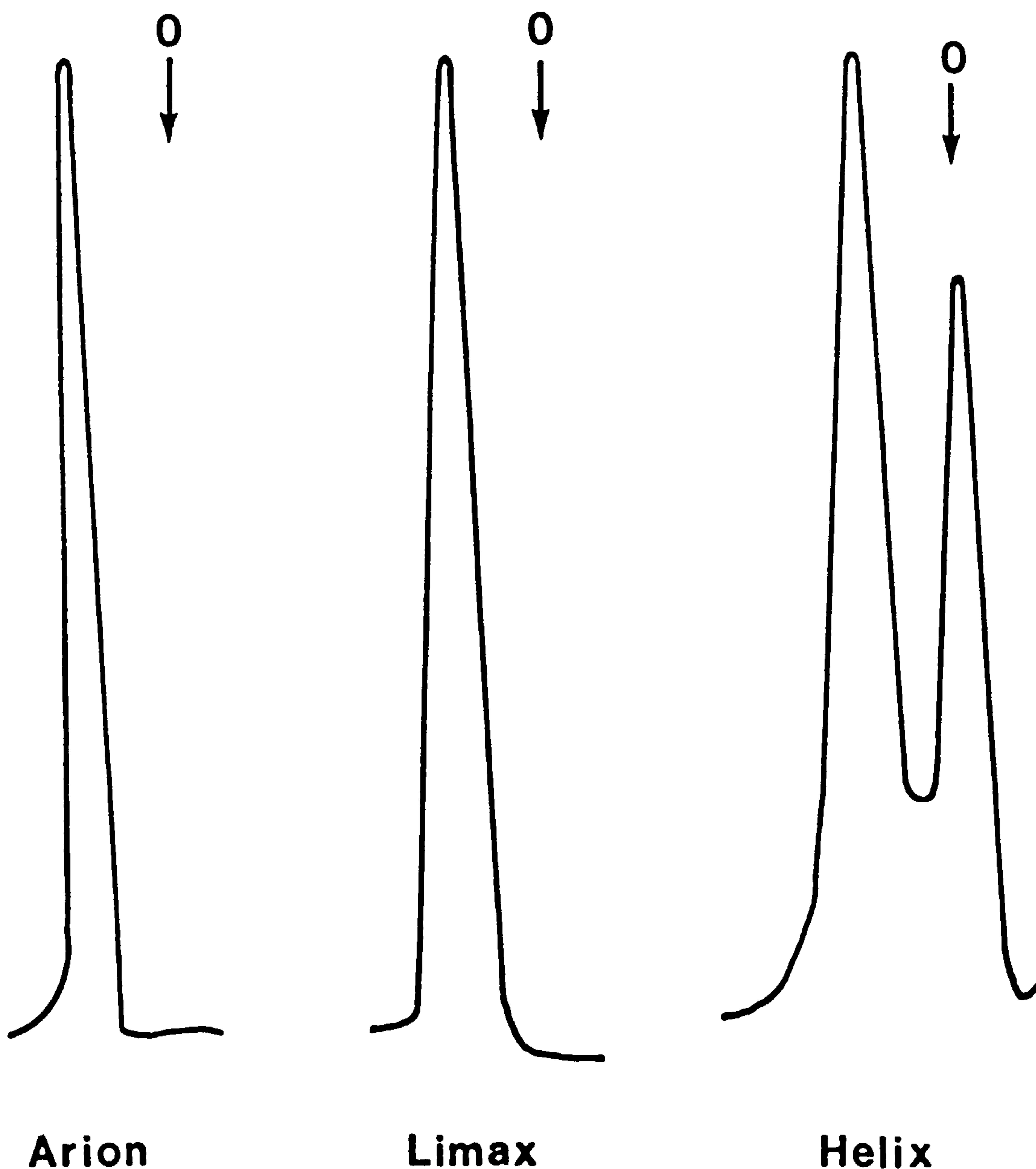




Figure 3.2.2.1.b)  $1/v$  against  $[i]$

$[i]$  = [salicaldehyde] ( $\mu\text{M}$ )  
 $s$  = [benzaldehyde] ( $\mu\text{M}$ )  
 $v$  = initial velocity ( $\text{nmol min}^{-1}$ )  
 Intercept of the plots at various  
 $s$  values =  $-K_i$ .  
 For full explanation see text.





**Figure 3.2.2.1.c) Cellulose acetate electrophoresis of aldehyde oxidase samples**

The samples were the 100,000g x 2 hours supernatant of digestive gland supernatant. Cellulose acetate foils (5cm x 7cm) were applied centrally and electrophoresed for 2 hours at 2.5mA per foil. After electrophoresis the foils were developed in an aldehyde oxidase specific dye (using benzaldehyde as the substrate). The results (as shown) were recorded using a recording densitometer. O represents the origin.

were performed with activity derived from *Arion ater* in which the concentration of salicylaldehyde (inhibitor,  $[i]$ ) was varied at four concentrations of benzaldehyde (substrate,  $s$ ). All assays were performed in duplicate. A plot of  $s/v$  against  $[i]$  (where  $v$  is the initial velocity) at various  $s$  values gave parallel lines (figure 3.2.2.1.a)). A plot of  $1/v$  against  $[i]$  at various  $s$  values gave a series of straight lines (figure 3.2.2.1.b)). The intercept of these lines gave an apparent  $K_i$  value at 15°C of  $0.12 \pm 0.02 \mu\text{M}$  ( $n=6$ ) (result is presented as the mean of the intercepts  $\pm$  standard deviation). The pattern of these two plots indicates that salicylaldehyde is a competitive inhibitor (Cornish-Bowden 1979). It is not surprising that this is the case as salicylaldehyde is very slowly oxidised by aldehyde oxidase from *Arion ater* (<3% the rate of benzaldehyde).

All the aldehyde oxidase activities could utilise the artificial electron-acceptor system of phenazine methosulphate (PMS) and thiazolyl-blue (MTT) which allowed an aldehyde oxidase specific insoluble dye to be made (see section 2.3.3.). Cellulose acetate electrophoresis of concentrated 100,000g x 2 hours supernatant of digestive gland homogenate revealed a single activity in *Arion* and *Limax*. Two "isoenzymes" were apparent in the *Helix* supernatant one of which had the same mobility as the *Arion* and *Limax* enzymes (see figure 3.2.2.1.c)). All of the enzymes could be demonstrated with benzaldehyde as substrate; the two *Helix* "isoenzymes" could be demonstrated with salicylaldehyde as substrate whereas the *Arion* and *Limax* enzymes could not. No staining was observed when a substrate-free dye control was used.



Tissue (n=4)	mg tissue / g snail	Aldehyde oxidase (specific activity) (nmol min <sup>-1</sup> mg protein <sup>-1</sup> )
Digestive gland	67.9 ± 9.5	2.45 ± 0.8
Kidney	11.2 ± 0.4	17.96 ± 1.7
Crop	5.4 ± 1.0	5.02 ± 2.6
Stomach + Intestine	19.1 ± 2.1	2.0 ± 0.72
Reproductive duct	20.9 ± 1.6	0.13 ± 0.05
Albumin Gland	100 ± 16.8	Not detected

**Table 3.2.2.2.a) Aldehyde oxidase activity in tissues of *Achatina fulica*.**

Activity was estimated by measuring hydrogen peroxide production using the TBHBA-AA-peroxidase linked assay. The results are presented in the form of the mean ± the standard deviation (n=4).

### 3.2.2.2. Aldehyde Oxidase Activity in the Tissues of *Achatina fulica*

Aldehyde oxidase activity could be detected in tissue homogenates of *Achatina fulica*. The tissue localisation of aldehyde oxidase was investigated using benzaldehyde as the substrate and measuring hydrogen peroxide production with the TBHBA-AA-peroxidase linked assay (see section 2.3.2.2.). The results are shown in table 3.2.2.2.a). As with alcohol oxidase in this species the highest specific activity was found in the kidney (see section 3.2.1.3.). Detectable activity was also found in all the other tissues with the exception of the albumin gland.

The 100,000g x 2 hours supernatant of digestive gland homogenate was used as a source of aldehyde oxidase activity. Digestive gland was chosen because of its relatively large size compared to the kidney. The activity was tested with a variety of aldehyde and heterocyclic potential substrates by measuring hydrogen peroxide production (at 30°C) using the TBHBA-AA-peroxidase linked assay. The results are shown in table 3.2.2.2.b). No activity was detectable with hypoxanthine (2mM) indicating the absence of xanthine oxidase. Purine (a substrate for mammalian aldehyde and xanthine oxidases) and 6-methyl-purine (mammalian aldehyde oxidase substrate) (Krenitsky *et al* 1972, 1974) at 2mM were not substrates; 2-hydroxy-pyrimidine was a moderate substrate at 2mM but a poor substrate at 0.2mM compared to benzaldehyde at these concentrations. Benzaldehyde, salicylaldehyde (inhibitory for *Arion ater* and *Limax flavus* aldehyde oxidase) and vanillin (3-methoxy-4-hydroxy-benzaldehyde) were good substrates at low concentration (0.2mM). At higher concentration (2mM) activity with vanillin and particularly salicylaldehyde was lower than expected when compared to activity with benzaldehyde at 2mM. It is possible that these hydroxy-

Substrate	0.2mM	2mM
Benzaldehyde	100%	100%
Salicylaldehyde	58%	20%
Vanillin	73%	59%
Dodecanal <sup>a</sup>	-	108%
Hypoxanthine	0	<2%
2-Hydroxy-pyrimidine	10%	46%
Purine	0	<2%
6-Methyl-purine	0	<2%

**Table 3.2.2.2.b) Activity of aldehyde oxidase from *Achatina fulica* with various substrates**

Activity with benzaldehyde was arbitrarily defined as 100% and activity is expressed as a percentage of that found with benzaldehyde at that concentration. Activity was estimated by measuring hydrogen peroxide generation using the TBHA-AA peroxidase linked assay.

<sup>a</sup> Dodecanal was at a concentration equivalent to a 40% saturated aqueous solution.



substituted substrates are somewhat inhibitory at higher concentrations. Dodecanal (*n*-dodecyl aldehyde) was assayed at a concentration equivalent to 40% saturation in aqueous solution and was found to be an excellent substrate despite its poor solubility.

Cellulose acetate electrophoresis (see section 2.3.3.) of the 100,000g x 2 hours supernatant of kidney and digestive gland homogenates indicated the presence of the same single "isoenzyme" in both tissues. This had the same mobility as the enzyme found in *Arion ater* and *Limax flavus* digestive gland (see section 3.2.2.1.) The possibility that the activity with dodecanal and benzaldehyde might be due to different enzymes was investigated by cellulose acetate electrophoresis. Digestive gland dodecanal oxidase had the same mobility as benzaldehyde oxidase indicating that both activities were probably due to the same enzyme. This view was confirmed by competition studies where activity with benzaldehyde and dodecanal supplied together was compared to activity with dodecanal and benzaldehyde supplied singly. The activities were found not to be additive when both substrates were supplied. Further evidence for the presence of a single enzyme was provided by the heat stability of both activities. Both benzaldehyde and dodecanal oxidase activities were stable at 48°C for 20 minutes but inactivated after 20 minutes at 56°C.

SUBSTRATE	SPECIES			
	<i>Arion ater</i>	<i>Limax flavus</i>	<i>Helix aspersa</i>	<i>Achatina fulica</i>
D-Mannitol	100% (15.8mM)	100% (13.8mM)	100% (9.2mM)	100% (19.0mM)
D-Arabitol	118% (53.0mM)	136% (21.1mM)	122% (16.5mM)	117% (7.6mM)
Sorbitol	19%	19%	26%	29% (190mM)
D-Xylitol	<2%	<2%	<2%	7%
D-Ribitol	<2%	<2%	<2%	<5%

**Table 3.2.3.1.- Properties of mannitol oxidase activity in subcellular preparations derived from the digestive glands of four species of gastropod mollusc.**

Activity was estimated by measuring hydrogen peroxide production using the TBHBA-AA-peroxidase linked assay. Substrates were at a concentration of 200mM. Activity with mannitol was arbitrarily defined as 100%. Numbers in parentheses are apparent Km values (by the Direct Linear Plot).

### 3.2.3. Mannitol Oxidase

#### 3.2.3.1. Mannitol Oxidase Activity in Four Species of Gastropod Mollusc

The peak mannitol oxidase fractions from isopycnic centrifugation experiments were used to investigate mannitol oxidase activity in four species of gastropod mollusc. The methods are as described in sections 2.2.2.5. (*Arion ater*, *Limax flavus* and *Helix aspersa*) and 2.2.2.2. (*Achatina fulica*). The results of typical fractionation experiments are shown in sections 3.1.3.1. (*Arion ater*, *Limax flavus* and *Helix aspersa*) and 3.1.2.1. (*Achatina fulica*). Activity was estimated using the TBHBA-AA-peroxidase linked assay to measure hydrogen peroxide production (see section 2.3.3.3.). Assays were performed at 23°C (*Arion ater*, *Limax flavus* and *Helix aspersa*) or 30°C (*Achatina*). The results are summarised in table 3.2.3.1..

There is very little difference between species; D-arabitol (at 200mM) is a marginally better substrate than D-mannitol for all four species. Apparent  $K_m$  values for D-mannitol are similar in all four species although there is more variation in the values for D-arabitol (see table 3.2.3.1.) The apparent  $K_m$  for sorbitol (D-glucitol) for the *Achatina fulica* enzyme is very high (190mM) suggesting that it is unlikely to be a physiological substrate. No activity was readily detectable with D-ribitol in any of the species and D-xylitol was slowly oxidised only by the enzyme from *Achatina fulica*.



## 4. DISCUSSION

## 4.1. The Subcellular Fractionation of Gastropod Digestive Gland

### 4.1.1. The Mannosome

The membrane structures associated with mannitol oxidase in *Arion ater* digestive gland P.N.S. have different sedimentation properties to other organelles in both rate-dependent and isopycnic sucrose-density gradients. Successive rate-dependent and isopycnic sucrose-density gradient centrifugation techniques have been used to isolate and partially purify the membrane structures that bear mannitol oxidase. The isolated membranes were examined by electron microscopy (see section 3.1.1.3.). These membranes have a distinctive tubular appearance and typically consist of seven tubules (each of approximately 50nm diameter) in a hexagonal 6 + 1 arrangement surrounded by a smooth membrane. S.D.S.-P.A.G.E. analysis of the partially purified membranes revealed the presence of a predominant polypeptide of 68-70 kDa which probably corresponds to mannitol oxidase (Vorhaben *et al* 1986). Fainter higher molecular weight bands were also visible and the presence of mitochondrial-membrane contamination was also indicated. The interim term mannosome has been proposed for these structures. Although mannitol oxidase is the only activity identified associated with these membranes it is unlikely that this is the sole function of such an elaborate structure. The phospholipid, carbohydrate and protein content as well as other characteristics of mannosomes awaits detailed investigation.

There have been numerous reports by electron microscopists of structures with a similar appearance and dimension in the tissues of terrestrial gastropods. Moya and Rallo (1975) observed these structures in cells of the digestive gland of *Arion empiricorum* (syn. *Arion ater*). The authors described the tubules as being within cisterns of the endoplasmic reticulum and therefore described the structures as intracisternal polycylinders (I.C.P.C.). It was postulated that the

tubules could be involved in ionic balance and water economy. They could possibly assist in the oxidative and excretory functions of the cell. Identical structures have also been observed by David and Götze (1963) in the digestive gland of *Arion hortensis* and by Czarna *et al* (1985) in the digestive gland of *Arion rufus* (syn. *Arion ater*).

Triebskorn and Köhler (1992) observed tubular mannosomes in the digestive gland of *Arion lusitanicus*. They considered these structures to be the result of infoldings of the smooth or degranulated endoplasmic reticulum. The unique polypeptide pattern seen in isolated mannosomes provides evidence against this view; the large number of proteins one would expect to see associated with the endoplasmic reticulum are absent in the isolated mannosomes and a single polypeptide predominates (probably mannitol oxidase). If the mannosomes are a specialised section of the endoplasmic reticulum a complex mechanism would be required to exclude the major endoplasmic reticulum proteins. Likewise a similar mechanism would be needed to exclude mannitol oxidase from the normal endoplasmic reticulum; subcellular fractionation experiments show that mannitol oxidase does not sediment with the endoplasmic reticulum marker enzymes NADPH-cytochrome c reductase and aromatic alcohol oxidase. The resolution of mannosomes from the endoplasmic reticulum in rate-dependent gradients (see sections 3.1.1.2. and 3.1.3.2.) in several species (*Arion ater*, *Arion lusitanicus* and *Helix aspersa*) also mitigates against the argument that mannosomes are only infoldings of the endoplasmic reticulum.

Triebskorn and Künast (1990) also observed very similar structures to mannosomes in cells of the intestine of the slug *Deroceras reticulatum* (syn. *Agriolimax reticulatum*; family : *Limacidae*) five hours after treatment with a lethal dose of the molluscicide cloethocarb. They described the structures as arising from the degranulated endoplasmic reticulum and were formed in response to poisoning. This study and others (e.g.: Moya and Rallo 1975, Triebskorn and Köhler 1992) indicate that mannosomes are unmodified organelles that are normally present in cells of terrestrial gastropods. Whether the mannosomes are absent in control specimens of *Deroceras reticulatum* (animals not subjected to treatment with cloethocarb) is not known. It is



conceivable that these structures were overlooked in control animals rather than being genuinely absent.

In both of the above reports (Triebkorn and Künast 1990, Triebkorn and Köhler 1992) the outer membrane of some mannosomes have reticulum-like extensions which are described as being continuous with the smooth endoplasmic reticulum and therefore provide evidence for the tubules being a specialised region of the endoplasmic reticulum. However there is no biochemical or strong morphological evidence for this view and the extensions could equally be continuous with the peroxisomal reticulum (Yamamoto and Fahimi 1987). It is also possible that these extensions form an independent "mannosomal reticulum" that serves to interconnect the bundles of tubules.

Analytical sucrose-gradient centrifugation techniques used in this study have indicated that the membranes associated with mannitol oxidase in the digestive gland of *Limax flavus* (also of the family *Limacidae*) have slightly different sedimentation properties to those of *Arion ater*, *Arion lusitanicus* and *Helix aspersa*. The mannitol oxidase-bearing membranes in *Limax flavus* digestive gland are therefore either morphologically different or react somewhat differently to homogenisation compared to those in the other species investigated (see section 3.1.3.). *Deroceras reticulatum* is a related species in which tubular mannosomes have been observed although only in animals treated with cloethocarb (Triebkorn and Köhler 1992). It would be interesting to investigate the sedimentation properties of mannitol oxidase-bearing membranes in the alimentary tissues of *Deroceras reticulatum* in both normal animals and following treatment with cloethocarb. This could possibly indicate if poisoning promotes the formation of mannosomes or whether they are normally present. If it is the case that poisoning with cloethocarb promotes the formation of mannosomes in alimentary tissues of *Deroceras reticulatum* the sedimentation properties of mannitol oxidase in poisoned animals should be similar to that of *Arion ater* mannitol oxidase whereas mannitol oxidase-bearing membranes from control animals would be expected behave similarly to those from untreated *Limax flavus* (see section 3.1.3.). Similarly it would be worthwhile repeating the analytical subcellular fractionation of *Limax flavus* digestive gland and comparing

the sedimentation of mannitol oxidase in both normal and cloethocarb-treated animals. These experiments would indicate whether mannosomes are formed only in response to stress conditions in this family of slugs or whether there are differences within the family.

Abolinš-Krogis (1970) reported the presence of structures virtually identical to mannosomes in the digestive gland of *Helix pomatia* following starvation and experimental shell damage. However in these stressed animals tubules were more commonly of larger diameter (approximately 100nm) and sometimes singular but often surrounded by six irregular or deflected tubules (described as micro-blebs). The cells of the digestive gland in these animals were considered to be in an "activated" state in order to regenerate the damaged shell.

This study has provided some evidence for the presence of tubular mannosomes in the digestive gland of *Helix aspersa* which have similar sedimentation properties (and therefore probably similar density, shape and dimensions) to those isolated from *Arion ater* digestive gland (see section 3.1.3.). The animals used had not been subjected to any stress conditions; they had not been starved nor had experimental shell damage been inflicted on them. *Helix aspersa* and *Helix pomatia* are closely related species therefore it is probable that the subcellular organisation would be similar in both species. The regular tubular structures (mannosomes) observed in those cells considered by Abolinš-Krogis (1970) to be in an "activated" state are therefore likely to be present in normal cells. However the less regular tubules also observed in the cells of *Helix pomatia* following shell damage are rather more interesting. It is conceivable that the regular patterned mannosomes are subject to a morphological change in stress situations such as that following extensive shell damage.

Cornejo (1987) reported the presence of tubular structures identical to mannosomes in the alimentary tissues the snail *Theba pisana* (family : *Helicellidae*). It was observed that these tubules increased in number following starvation. A parallel increase in peroxisomes in starved animals prompted Cornejo to speculate that the tubular organelles may be related to the tubular peroxisomes studied by Gorgas and Völkl (1984) in the mouse meibomian gland. These



tubular peroxisomes are of a comparable diameter (on average approximately 50nm in diameter) to the individual tubules in mannosomes but do not have a surrounding smooth membrane. Tubular peroxisomes progressively decrease in catalase activity following morphological alteration during cell differentiation.

Unusual aggregations of tubules have been reported in several species of marine and freshwater gastropod. McLean (1978) reported tubules in the digestive gland of the sea-slug *Alderia modesta*. The tubules were approximately 60nm in diameter and often seen hexagonally packed in bundles of more than 50 individual tubules. Adjacent tubules appeared to be linked via two indistinct granules. The tubules were observed in normal individuals and it was suggested that they were an elaboration of the endoplasmic reticulum. Kessel and Beams (1984) reported the presence of unusual tubules in the reproductive tract of the fresh-water snail *Limnaea stagnalis*. These tubules were also hexagonally packed in large groups although the individual tubules were of a smaller diameter (28nm). Their appearance is very like that of the tubules observed in *Alderia modesta* and similarly possess fine interconnections. Kitajima and Paraense (1983) observed bundles of tubules in the spermatheca of virgin specimens of the freshwater snail *Biomphalaria glabrata*. The individual tubules were approximately 60nm in diameter and hexagonally packed in moderately sized bundles (15-50 individual tubules); although found in larger groups the appearance of these structures is very like the mannosomes observed in *Arion ater*. Both of these reports suggested that the tubules were associated with the endoplasmic reticulum.

Tubules of 100nm diameter have been observed in the amoebocytes of *Helix pomatia* and are described as tubular endoplasmic reticulum (Abolinš-Krogis 1972). In contrast to the structures previously described by the same author (1970) in the digestive gland of this animal the amoebocyte tubules are nearly always singular rather than in groups with smaller (20nm) connecting tubules. They have a very distinctive and regular six-pointed star appearance in the electron micrographs. This regularity is in contrast to the singular tubules also reported in the digestive gland of this species (Abolinš-Krogis 1970).



Tubular organelles have also been reported in non-gastropod species. For example the tubular peroxisomes in mouse meibomian gland (Gorgas and Völkl 1984) mentioned above. Tubules of approximately 80nm diameter have been observed in normal hamster liver (Jones and Fawcett 1966). The authors could not be sure whether these tubules were local specialisations of the smooth endoplasmic reticulum or were entirely independent structures. Tubules of between 30nm and 100nm have been reported in the chloride-cells of the gill epithelium of the salt-water fish *Oligocottus maculosus* osmotically stressed in diluted sea-water. Hexagonally packed bundles of tubules of 80nm diameter have been observed in the haemocytes of the insect *Periplaneta americana* (Baerwald and Boush 1970).

The extent to which the structures listed above are related is as yet unknown. Mannitol oxidase-bearing mannosomes have been positively identified in *Arion ater*; centrifugal evidence for their presence in *Arion lusitanicus* and *Helix aspersa* is very strong. It is not known whether the virtually identical structures observed by electron microscopists in *Helix pomatia*, *Theba pisana* and *Deroceras reticulatum* are associated with mannitol oxidase activity but it is very likely that these structures are functionally similar to the mannosomes of *Arion ater*. One very interesting area of future investigation would be to study the effect of stress conditions on mannosomes. Cornejo (1987) suggested that tubular structures in the alimentary tissues of *Theba pisana* increased following starvation; if there is an increase in mannosomes during starvation would a concomitant increase in mannitol oxidase activity be observed in this species? The observation of Abolinš-Krogis (1970) of tubular structures in *Helix pomatia* following shell-damage also suggests future possible experiments. It is very likely that mannosomes are present in normal specimens of the related species *Helix aspersa*; subcellular fractionation of the digestive gland of animals at varying periods following extensive experimental shell damage would indicate whether the mannitol oxidase-bearing membranes change their sedimentation properties under stress conditions. The effect of molluscicides on the sedimentation properties of mannosomes in various species would also be an area for future investigation.

The mannosomes isolated from *Arion ater* digestive gland are elaborate structures and it is difficult to believe that their sole function is mannitol oxidation. The fate of the hydrogen peroxide generated by mannitol oxidase is unknown; it appears likely from the subcellular fractionation experiments that they lack catalase activity (see section 3.1.1.3.). It is possible that it permeates into the surrounding cytosol where it could be detoxified by glutathione peroxidase and cytosolic catalase. The search for additional functions of the mannosomes would seem to be necessary. Although the membranes have a predominant protein of 68-70kDa other fainter bands were also visible in the S.D.S.-P.A.G.E. analysis of isolated mannosomes. It cannot be ruled out that mannitol oxidase is a multifunctional protein. As the predominant protein it is possible that the putative mannitol oxidase polypeptide (68-70 kDa) plays a structural role in the mannosome membranes.

Tubular structures have also been observed in marine and fresh-water gastropods which are rather different from the mannosomes found in terrestrial species. The presence of mannitol oxidase activity in these tubules would go some way to proving that they are members of the same organelle family. If mannitol oxidase is detectable in tissues of aquatic and marine gastropods the results of subcellular fractionation experiments could provide evidence for such a relationship. However the response to homogenisation and the sedimentation properties of these larger tubule bundles would be hard to predict.

Using a highly purified mannitol oxidase preparation or the 68kDa protein eluted from S.D.S.-P.A.G.E. gels of isolated mannosomes from *Arion ater* antibodies could be raised in a mammalian species (for example sheep or rabbit). These antibodies could be used to search for immunologically cross-reacting material in species of gastropod that do not express mannitol oxidase activity using techniques such as Western-blotting and immunohistochemistry (at both light- and electron-microscopy magnification levels). As mannitol oxidase activity is probably an adaption to a herbivorous diet it is unlikely to be found in carnivorous terrestrial species such as the *Testacella* slugs. However it is possible that the subcellular organisation in such species is similar



to phylogenetically related herbivorous species. Protein from species that lack mannitol oxidase activity which cross-reacts immunologically with antibody to mannitol oxidase from herbivorous species would indicate the presence of a related protein that is not functionally a mannitol oxidase but performs another essential function, for example as a structural protein. Electron-microscopic immunohistochemistry using anti-mannitol oxidase antibodies could indicate the subcellular localisation of immunologically cross-reacting proteins. This could potentially bring to light related mannosome-like structures even where mannitol oxidase activity is absent and the subcellular fractionation techniques described in this thesis were unsuccessful. Immunological techniques could also be used to determine the relatedness of the tubular structures observed in marine and fresh-water molluscs.

The predominant 68-70 kDa polypeptide (possibly mannitol oxidase) is probably not the only protein associated with these membranes. It would therefore be worthwhile to raise antibodies to the isolated whole organelle. Obtaining such a highly purified preparation of mannosomes would however present some difficulty as it would be necessary to remove virtually all the mitochondrial-membrane contamination that co-sediments with the mannosomes (as indicated by S.D.S.-P.A.G.E. analysis of isolated mannosomes, see section 3.1.1.3.). Antibodies raised to the whole organelle could be used in much the same way as those specific for mannitol oxidase but as they would recognise a range of mannosomal proteins there could be a greater probability of detecting an immunologically cross-reacting protein in the tubular structures observed in various gastropods.

The investigation could be extended to include tubular structures such as those observed in insects and mammals. The presence of a hydrogen peroxide-generating mannitol oxidase and the observation of Cornejo (1987) that mannosomes increase in parallel to peroxisome proliferation in at least one species of snail suggest a possible relationship with the tubular peroxisomes of Gorgas and Völkl (1984). Significantly these tubular peroxisomes progressively lose their catalase activity therefore the absence of catalase activity in mannosomes does not necessarily preclude a possible connection with the peroxisomal system within the gastropod cell. The tubular



peroxisomes of mouse meibomian gland are involved in the biosynthesis of glycerol-ether phospholipids and wax esters. The first step in the biosynthesis of both of these lipid types is the reduction of fatty-acyl-Coenzyme A to the corresponding fatty alcohol. The enzyme responsible for this reduction in mammals, fatty-acyl-Coenzyme A reductase (long-chain alcohol forming) is NADPH-specific and catalyses a four-electron transfer without the release of an intermediate aldehyde. The enzyme is localised on the outer surface of the peroxisomal membrane (cytosol side) in guinea pig intestine mucosal cells (Burdett *et al* 1991). Wax-esters are found in high levels in mammalian sebaceous glands. Gorgas and Völkl (1984) suggested that the increase in peroxisomal membrane area that occurs with the formation of tubular peroxisomes indicates an increased wax-ester production. The digestive gland of *Arion ater* and other gastropods contains unusually high levels of glycerol-ether phospholipids (Thompson 1963). It is tempting to speculate that the mannosomes may be involved in the biosynthesis of this type of lipid and this offers another possible connection with the tubular peroxisomes of the mouse meibomian gland. Tubular structures have been observed in other mammalian sebaceous glands, for example the sebaceous glands of the primate *Galago crassicaudatus* (Bell 1970). Immunological techniques could illustrate a relationship between the tubular mannosomes of terrestrial gastropods and apparently unrelated tubular phenomenon observed in other phyla.

#### 4.1.2. The Subcellular Localisation of Aromatic Alcohol Oxidase

Both aromatic alcohol oxidase and mannitol oxidase generate hydrogen peroxide but surprisingly neither are to be found in the peroxisomes which would be the most obvious localisation for such membrane-bound activities. The localisation of mannitol oxidase has been considered in detail above. Aromatic alcohol oxidase is most likely localised in the endoplasmic reticulum of gastropods (although the localisation was rather more obscure in *Limax flavus*, see section 3.1.3.). Generation of hydrogen peroxide is a normal function of the endoplasmic reticulum although much of this is via the cytochrome P450 system (see section 1.2.1.3.). It is probable that the hydrogen peroxide generated by aromatic alcohol oxidase is destroyed by the strongly reducing environment of the cytosol which generally contains high levels of glutathione peroxidase and reduced glutathione. (see section 1.2.2.2.). A search for enzyme activities that utilise the hydrogen peroxide produced (for example specific peroxidases) would seem worthwhile.

Substrate	Species	
	<i>Arion ater</i>	<i>Pleurotus eryngii</i>
Cinnamyl alcohol	100%	100%
2-Naphthalene methanol	79%	168%
4-Methoxybenzyl alcohol	62%	127%
3-Methoxybenzyl alcohol	60%	22%
2-Methoxybenzyl alcohol	<2%	<1%
Crotyl alcohol	40%	-
2,4-Hexdien-1-ol	-	117%
Coniferyl alcohol	14%	<1%
2-Phenylethanol	-	<1%
2-Naphthalene ethanol	<2%	-
4-Hydroxybenzyl alcohol	<2%	<1%
Km for 4-methoxybenzyl alcohol	184μM	40μM
pH optimum	6.6	6.0-6.5

**Table 4.2.1.1. - Comparison of the substrate specificities of aromatic alcohol oxidase from *Pleurotus eryngii* and *Arion ater*.**

The data for *Pleurotus eryngii* is taken from Guillen *et al* (1990 and 1992). The data for *Arion ater* is as described in sections 3.2.1.1. and 3.2.1.2. of this thesis. Activity with cinnamyl alcohol is arbitrarily defined as 100 %.



## 4.2. Characterisation of Catalytic Activities

### 4.2.1. Aromatic Alcohol Oxidase

#### 4.2.1.1. Aromatic Alcohol Oxidase Activity in *Arion ater* and Other Temperate Gastropod Species

Molluscan aromatic alcohol oxidase is a particulate activity which has been demonstrated in several species of terrestrial gastropod in this study. The activity could be solubilised in a variety of non-ionic detergents. Solubilised activity was found to be active with aromatic methanols, cinnamyl alcohol and the  $\alpha$ - $\beta$ -unsaturated aliphatic crotyl alcohol (2-buten-1-ol) (see sections 3.2.1.1. and 3.2.1.2.). The activity from *Arion ater* has been the most extensively studied.

The substrate specificity of the solubilised *Arion ater* activity is surprisingly similar to that of soluble fungal aromatic alcohol oxidases particularly the enzyme derived from *Pleurotus eryngii* (Guillen *et al* 1992) (see table 4.2.1.1.). Both the *A. ater* and *P. eryngii* enzymes lack activity with 2-methoxy-benzyl alcohol although aromatic alcohol oxidase derived from other gastropod species (*Helix aspersa* and *Limax flavus*, see section 3.2.1.2.) can oxidise this substrate. The presence of a hydroxyl group markedly affects the suitability of the substrate. 4-Hydroxybenzyl alcohol is not a substrate for either enzyme; it is also not utilised by the enzyme from *Pleurotus sajor-caju* (Bourbonnais and Paice 1988) and only poorly oxidised by activities from *Bjerkandera adusta* (Muheim *et al* 1990) and *Coriolus (Polystictus) versicolor* (Farmer *et al* 1960). Coniferyl alcohol (3-methoxy-4-hydroxy-cinnamyl alcohol) is poor substrate for the *Arion ater* enzyme (it is utilised at only 14% the rate of the unsubstituted cinnamyl alcohol). The effect of hydroxy-substitution of cinnamyl alcohol is also seen with fungal enzymes; coniferyl alcohol is oxidised at only 20% the rate of cinnamyl alcohol by the enzyme derived from

*Pleurotus ostreatus* (Sannia *et al* 1991) and is not a substrate for aromatic alcohol oxidase from *Pleurotus eryngii* (Guillen *et al* 1992) and *Pleurotus sajor-caju* (Bourbonnais and Paice 1988). A similarity is also seen in the inability of the *Arion ater* enzyme and certain fungal enzymes (eg : *Pleurotus eryngii* (Guillen *et al* 1992) and *Bjerkandera adusta* (Muheim *et al* 1990)) to oxidise aromatic ethanols.

Molluscan aromatic alcohol oxidases from all the species investigated are able to oxidise the  $\alpha$ - $\beta$ -unsaturated crotyl alcohol (2-buten-1-ol) (see section 3.2.1.2). Aromatic alcohol oxidase from *Pleurotus eryngii* is able to oxidise the  $\alpha$ - $\beta$ -unsaturated 2,4-hexadien-1-ol (Guillen *et al* 1992). This ability to utilise  $\alpha$ - $\beta$ -unsaturated aliphatic alcohols is shared by an alcohol oxidase from the plant *Tanacetum vulgare* although this enzyme is unable to utilise aromatic substrates (Banthorpe *et al* 1976). Other fungal aromatic alcohol oxidases cannot oxidise these alcohols; for example the *Pleurotus sajor-caju* enzyme cannot oxidise crotyl alcohol (2-buten-1-ol) or allyl alcohol (2-propen-1-ol) (Bourbonnais and Paice 1988) and the *Pleurotus ostreatus* enzyme is unable to oxidise allyl alcohol (Sannia *et al* 1991).

4-Methoxy-benzyl alcohol (anisyl alcohol) is a favoured substrate for all the molluscan aromatic alcohol oxidases studied and also for all the reported fungal enzymes. The apparent  $K_m$  values for 4-methoxy-benzyl alcohol are comparable with enzyme from several sources : *Pleurotus eryngii* (40 $\mu$ M) (Guillen *et al* 1992), *Bjerkandera adusta* (240 $\mu$ M) (Muheim *et al* 1990) and *Arion ater* (184 $\mu$ M).

An interesting point is the acceptability of halogenated aromatic alcohols to both fungal and molluscan enzymes. 3-Fluorobenzyl alcohol was found to be an excellent substrate second only to cinnamyl alcohol (at 1.7mM) in this study for the *Arion ater* enzyme. 3-Chloro-4-methoxy-benzyl alcohol and 3,5-dichloro-4-methoxy-benzyl alcohol are excellent substrates for aromatic alcohol oxidase from *Bjerkandera* sp. strain BOS55 (De Jong *et al* 1994). These chloro-substituted aromatic alcohols are synthesised *de novo* by the fungus and are believed to be produced as a means of generating extracellular hydrogen peroxide via aromatic alcohol oxidase. As these chloro-substituted benzyl alcohols are preferentially oxidised by aromatic alcohol oxidase they



also prevent unwanted oxidation of veratryl alcohol (which is also synthesised *de novo* to protect lignin peroxidase from inactivation, see section 1.3.5.). This is unlikely to be the role of the *Arion ater* enzyme. The greater activity found with halogenated-benzyl alcohols is likely to be a function of the electron-withdrawing effect of the halogen making the hydroxy-methyl group more reactive. This property is exploited by *Bjerkandera* sp. strain BOS55 but is probably coincidental (ie : of no physiological importance) for *Arion ater*.

The pH optima of aromatic alcohol oxidases from various sources are also somewhat similar. Particulate *Arion ater* activity has a pH optimum of 6.6. All the reported fungal soluble aromatic alcohol oxidases have slightly acid pH optima (pH 5.0 - 6.8) (see section 1.3.5.). The *Arion ater* enzyme shows greater than 80% of its maximal activity (at pH 6.6) at pH 10.0. This is in contrast to several fungal enzymes that are intolerant of alkaline conditions (eg : *Polystictus versicolor* (Farmer *et al* 1960) and *Pleurotus eryngii* (Guillen *et al* 1990)). Several fungal enzymes (eg : *Pleurotus eryngii* (Guillen *et al* 1990) and *Pleurotus sajor-caju* (Bourbonnias and Paice 1988)) are active at pH 3.0 whereas the *Arion ater* enzyme is completely inactive at this pH.

Recent research with *Pleurotus eryngii* (Guillen *et al* 1994, Guillen and Evans 1994) has shown that the main function of aromatic alcohol oxidase is the generation of extracellular hydrogen peroxide. Hydrogen peroxide is essential for lignin biodegradation and is utilised by extracellular lignin peroxidase and manganese-dependent peroxidase. Aromatic alcohols such as veratryl alcohol and anisyl (4-methoxybenzyl) alcohol are oxidised to their corresponding aldehydes by extracellular aromatic alcohol oxidase with the concomitant generation of hydrogen peroxide. Aromatic aldehydes are also oxidised to their corresponding acids by this enzyme but to a much lesser extent. Intracellular NADPH-dependent aldehyde and acid reductases reduce the products of aromatic alcohol oxidase back to the alcohol. This system acts as a redox-cycle to continually provide substrates for the oxidase and so generate extracellular hydrogen peroxide from intracellular NADPH. Aromatic alcohols and aldehydes are the likely breakdown products of lignin. However the substrates for



aromatic alcohol oxidase are known to be synthesised *de novo* by various fungi. For example the major aromatic extracellular metabolite of fungi of the *Pleurotus* genus is anisaldehyde (4-methoxybenzaldehyde) (Guillen *et al* 1994) and chloro-substituted anisyl alcohols are known to be synthesised by a *Bjerkendera* strain (De Jong *et al* 1994). The lignin monomeric alcohol coniferyl alcohol is either a poor substrate or not a substrate at all for fungal aromatic alcohol oxidases. This supports the view that aromatic alcohol oxidase does not function primarily to oxidise the products of lignin degradation.

The molluscan enzyme is intracellular and membrane-bound; it is probably localised on the endoplasmic reticulum (see section 3.1.1.). The function of the molluscan enzyme is therefore likely to be different to that of the fungal enzymes. Neuhauser *et al* (1978) reported that soil invertebrates (including the slug *Deroceras reticulatum* and the snail *Oxychilus draparnaldi*) were incapable even with their gut micro-organisms of the demethylation or degradation of lignin model compounds. However these animals could demethylate methoxylated aromatic compounds and degrade fully several aromatic compounds including vanillin and cinnamic acid. It is unknown to what extent gut micro-organisms play a part in this degradation. It was demonstrated by Neuhauser and Hartenstein (1976) that the gastropods *Arion hortensis* and *Oxychilus draparnaldi* have O-demethylase activity that is not due to gut micro-organisms. This activity cleaves the methyl-groups from methoxylated aromatic compounds and the methyl carbon is converted to carbon dioxide.

Plant matter consumed by herbivorous gastropods contains many aromatic compounds. For example the aromatic alcohols that are the precursors of lignin (coumaryl, coniferyl and sinapyl alcohols) are stored in plant tissues as the 4-O- $\beta$ -D-glucopyranoside prior to lignification (Savidge and Udagama-Randeniya 1992). It would seem worthwhile demonstrating that terrestrial gastropods have enzyme activities capable of the hydrolysis of these compounds. It is possible that aromatic alcohol oxidase and cytosolic aldehyde oxidase convert lignin precursors to their corresponding acids which can then be demethylated and further degraded.

Aromatic alcohol oxidase from all the temperate species investigated was able to oxidise the  $\alpha$ - $\beta$ -unsaturated aliphatic crotyl alcohol (2-butene-1-ol, *cis/trans* mixed isomers) at 40-60% the rate of cinnamyl alcohol. The alcohol oxidase described in the plant *Tanacetum vulgare* (Banthorpe *et al* 1976) is most active with  $\alpha$ - $\beta$ -unsaturated alcohols of various chain-length but also with medium- to long-chain C<sub>6</sub>-C<sub>12</sub>) saturated aliphatic alcohols.  $\alpha$ - $\beta$ -Unsaturated aliphatic alcohols are commonly found in plants. These include olefinic terpene alcohols that are components of essential plant oils. Geraniol (3,7-dimethyl-*trans*-2,*trans*-6-octadien-1-ol) is found in many plant oils including rose and lemon grass. Farnesol (3,7,11-trimethyl-*trans*-2,*trans*-6,*trans*-10-dodecatrien-1-ol) and nerol (3,7-dimethyl-*cis*-2,*trans*-6-octadien-1-ol) are also examples of  $\alpha$ - $\beta$ -unsaturated alcohols found in plant oils (Merck Index, 10th Edition). All of the above are substrates for the alcohol oxidase from *Tanacetum vulgare* (Banthorpe *et al* 1976). Although these potential substrates have not been tested with molluscan aromatic alcohol oxidase it is possible that these compounds are acceptable substrates as the  $\alpha$ - $\beta$ -unsaturated crotyl alcohol is readily oxidised. It is conceivable that aromatic alcohol oxidase and aldehyde oxidase convert these terpene alcohols into fatty acids which are then further metabolised.

In this study the  $\alpha$ - $\beta$ -unsaturated alcohols used (crotyl, cinnamyl and coniferyl alcohols) have been the commercially available products. In all cases the alcohols have been a mixture of the *cis* and *trans* isomers. It is therefore not known whether only one or both isomers are substrates. It would be interesting to investigate the effect of the configuration of the double bond on enzyme activity. However the kinetic parameters determined for these alcohols in this study must be viewed with this consideration in mind.

Aromatic alcohol oxidase activity was compared in three species of British gastropod (see section 3.2.1.2.). The activities are quite similar with cinnamyl alcohol (1mM) being the best substrate of those tested. There are two notable differences between the various species. Firstly amino-substituted benzyl alcohols are excellent substrates for the *Helix aspersa* enzyme with activity equivalent to that with cinnamyl alcohol.



However these alcohols were poor to moderate substrates for the *Limax flavus* and *Arion ater* activities. The *Arion ater* enzyme could not utilise 2-methoxybenzyl alcohol although both the *Limax flavus* and the *Helix aspersa* enzymes found this to be an acceptable substrate. Overall the *Helix aspersa* enzyme had the broadest substrate specificity.

Solubilised crude preparations of molluscan aromatic alcohol oxidase from several species have provided information regarding the substrate preferences of these unusual activities that appear to be unique in the animal kingdom. However very little information is known about the protein responsible for the observed enzyme activity. Future work would include purifying as fully as possible the enzyme from one or more sources. Purification to homogeneity would provide material that could be used to elucidate the presence of a prosthetic group (if any), whether the enzyme is monomeric or has multiple subunits and the molecular weight of the protein (and its subunits if applicable).

#### 4.2.1.2. Alcohol Oxidase Activity in *Achatina fulica*

The alcohol oxidase activity found in the large tropical snail *Achatina fulica* oxidises many of the substrates that are oxidised by other molluscan aromatic alcohol oxidases (see section 3.2.1.3.) including cinnamyl alcohol and crotyl alcohol (2-buten-1-ol). Crotyl alcohol (10mM) was the best substrate of those tested although cinnamyl alcohol (10mM) was oxidised at 74% the rate of crotyl alcohol. Again *cis/trans* mixed isomers were used in experiments and it is not known whether both isomers are substrates.

The *Achatina fulica* enzyme could also oxidise many saturated aliphatic primary alcohols unlike the temperate gastropod species investigated. Of those tested only methanol was found not to be a substrate. Ethanol (C<sub>2</sub>) up to hexadecan-1-ol (C<sub>16</sub>) unbranched alcohols were acceptable substrates. This activity is unusual in its



broad substrate specificity. The long-chain alcohol oxidases of *Candida tropicalis* (Kemp *et al* 1988) and *Simmondsia chinensis* (Moreau and Huang 1979) are dedicated fatty alcohol oxidases most active with C<sub>8</sub>-C<sub>16</sub> substrates (see section 1.3.5.). Amyl alcohol (100mM) is oxidised at only 33% the rate of crotyl alcohol (10mM) however it has a much lower apparent K<sub>m</sub> (41μM compared to 252μM for crotyl alcohol) suggesting that saturated alcohols are possible physiological substrates. Also unlike the *Arion ater* activity the aromatic ethanol 2-phenylethanol was an acceptable substrate.

Potentially a herbivorous gastropod would encounter many alcohols in its diet. Sources of aromatic and olefinic terpene alcohols have been discussed in the previous section (4.2.1.1.). The sources of these dietary alcohols apply equally to *Achatina fulica*. Saturated and other unsaturated alcohols would also be ingested. Ethanol would be present in partially decomposed or fermented fruit. Hexan-1-ol is present as the acetate ester in the seeds and fruit of some *Umbelliferae* species; hex-*cis*-3-ene-1-ol (leaf alcohol) and the corresponding aldehyde are found in the leaves of many odoriferous plants (Merck Index, 10th Edition). Fatty alcohols are likely to be encountered in the diet as wax-esters on the surface of leaves or in seeds and fruits. The action of alcohol oxidase and aldehyde oxidase (see section 3.2.2.2.) would conceivably convert a diverse range of ingested alcohols and aldehydes into their corresponding carboxylic acids prior to further metabolism.

Although a digestive role is likely for this enzyme the kidney has a higher specific activity of both alcohol and aldehyde oxidase (see sections 3.2.1.3. and 3.2.2.2.). This suggests an excretory role for the enzymes. Further investigation is required to elucidate the fate of ingested alcohol substrates. This could include measuring the levels and type of carboxylic acids excreted in the urine.

## 4.2.2. Aldehyde Oxidase

Molluscan aldehyde oxidase is a cytosolic hydrogen peroxide generating activity. Many of the aldehydes that are the likely products of aromatic alcohol oxidase activity are substrates for this enzyme. It is possible that the concerted action of aromatic alcohol oxidase and aldehyde oxidase convert a range of ingested alcohol and aldehyde compounds into their corresponding carboxylic acids prior to further metabolism (see sections 4.2.1.1. and 4.2.1.2. for a full discussion on the possible roles of these enzymes).

Activities from the digestive glands of *Arion ater*, *Limax flavus* and *Helix aspersa* were compared with a mammalian source of aldehyde oxidase (mouse liver) using a variety of aromatic aldehyde substrates. Xanthine oxidase activity could not be detected in any of the samples (see section 3.2.2.1.). The sample derived from mouse liver was fairly similar to the molluscan enzymes with respect to its activity with aromatic aldehydes although it was inhibited by 3-hydroxybenzaldehyde. The apparent  $K_m$  values obtained for each of the samples were comparable : 5.1 $\mu$ M (mouse), 1.9 $\mu$ M (*Arion ater*), 11.9 $\mu$ M (*Limax flavus*) and 5.5 $\mu$ M (*Helix aspersa*). Salicylaldehyde was a potent competitive inhibitor of the aldehyde oxidase activities from both *Limax flavus* and *Arion ater* (but not *Helix aspersa*). This similarity between the two slug species is surprising as *Limacidae* and *Arionidae* slugs are believed to be descended from different families of snails (South 1992).

Two isoenzymes of aldehyde oxidase were detected by cellulose acetate electrophoresis in the digestive gland of *Helix aspersa*. It is not known whether the two isoenzymes have similar substrate preferences but both could oxidise benzaldehyde and salicylaldehyde. The substrate comparisons and the apparent  $K_m$  value for benzaldehyde obtained with *Helix aspersa* sample must be viewed bearing in mind the possibility that two non-identical enzymes may be responsible for the observed activity.

Aldehyde oxidase from *Achatina fulica* digestive gland has also been investigated. This enzyme could oxidise aromatic as well as fatty (aliphatic) aldehydes that are the possible products of alcohol oxidase from the same source. Cellulose acetate electrophoresis and heat stability experiments indicated that a single enzyme was responsible for the oxidation of aromatic and aliphatic aldehydes.

It has been suggested that aldehyde oxidation is not a significant function of mammalian hepatic aldehyde oxidase but rather that aldehyde oxidase and xanthine oxidase represent a pair of substituted pyrimidine and purine oxidases (Krenitsky *et al* 1972). The *Achatina fulica* enzyme preparation was inactive with some purines that are excellent substrates for the mammalian enzyme and showed only limited activity with 2-hydroxy-pyrimidine. Therefore it is reasonable to conclude that this activity *in vivo* functions as an aldehyde oxidase rather than an oxidase of heterocycles. In this respect it is similar to an aldehyde oxidase from another invertebrate source (the crayfish, *Cambarus bartoni*) which is also inactive with mammalian aldehyde oxidase heterocyclic substrates (Hartenstein 1973). The crayfish enzyme has low  $K_m$  values for aromatic aldehydes (2 $\mu$ M for 2-aminovanillin and 3 $\mu$ M for vanillin) that are comparable to the low values found with molluscan enzymes for benzaldehyde (2-12 $\mu$ M).



### 4.2.3. Mannitol Oxidase

Hydrogen peroxide generating mannitol oxidase is an enzyme activity that appears to be unique to terrestrial gastropods (see section 1.5.3.). The enzyme is localised mainly in alimentary tissues and probably fulfils a digestive role (Vorhaben *et al* 1984). The enzyme is membrane-bound and this study has shown that it is probably localised in a specialised tubular organelle (mannosome) in at least one species (*Arion ater*) (see sections 3.1.1.3. and 4.1.1.).

D-Mannitol and other sugar alcohols are found in plants and are important storage carbohydrates in many fungi (Lewis and Smith 1967). Many terrestrial gastropods are fungivores (Neuhauser and Hartenstein 1976) and D-mannitol would represent a valuable nutritional resource. Mannitol oxidase is therefore a useful adaptation to a diet consisting of plants and fungi.

The activities from *Arion ater*, *Limax flavus*, *Helix aspersa* and *Achatina fulica* are very similar with regard to substrate specificity. Significant activity is found only with D-mannitol, D-arabitol and to a lesser extent with sorbitol. This suggests that the enzyme activity (although not necessary the protein) is well conserved in these animals. This implies that the function may be important to the maintenance of the animals. It would be interesting to test related carnivorous species (for example *Testacella* sp. slugs) for mannitol oxidase to see if the activity is retained despite a non-herbivorous diet (see also section 4.1.1.).

Vorhaben *et al* (1986) found an apparent  $K_m$  for D-mannitol of 6.9mM using solubilised and partially purified *Helix aspersa* mannitol oxidase and a peroxidase-linked assay to measure hydrogen peroxide production. This compares well with the values obtained in this study also using a peroxidase-linked assay but using subcellular fractions as the source of enzyme activity (ie : crude particulate enzyme) :- 9.2mM (*Helix aspersa*), 13.8mM (*Limax flavus*), 15.8mM (*Arion ater*) and 19.0mM (*Achatina fulica*).

The apparent  $K_m$  values are all in the millimolar range. Many other sugar alcohol oxidising enzymes also have relatively high  $K_m$  values. Mannitol dehydrogenase from *Aspergillus parasiticus* has a  $K_m$  for D-mannitol of 100mM (Niehaus and Dilts 1982); sheep liver sorbitol dehydrogenase has  $K_m$  values of 7.9mM for sorbitol and 91mM for D-mannitol (Walsall *et al* 1978). Sorbitol oxidase from apple leaves has a  $K_m$  for sorbitol of 100mM (Yamaki 1980); cytochrome-linked sorbitol dehydrogenase from *Gluconobacter suboxydans* has a  $K_m$  for sorbitol of 30mM (Shinagawa *et al* 1982). Molluscan mannitol oxidases therefore have apparent  $K_m$  values that compare favourably with the  $K_m$  values for many other sugar alcohol oxidising enzymes.

# BIBLIOGRAPHY



## BIBLIOGRAPHY

Abolinš-Krogis, A. (1970) Alterations in the fine structure of cytoplasmic organelles in the hepatopancreatic cells of the shell-regenerating snail, *Helix pomatia*. Z. Zellforsch. 108 : 516-529.

Abolinš-Krogis, A. (1972) The tubular endoplasmic reticulum in the amoebocytes of the shell-regerating snail *Helix pomatia*. Z. Zellforsch. 128 : 58-68.

Ameyama, M. and Adachi, O. (1982a) Alcohol dehydrogenase from acetic acid bacteria, membrane-bound. In : Wood, W.A. (Ed.) Methods in Enzymology vol. 89 (Academic Press, New York) pp.450-457.

Ameyama, M. and Adachi, O. (1982b) Aldehyde dehydrogenase, acetic acid bacteria. In : Wood, W.A. (Ed.) Methods in Enzymology vol. 89 (Academic Press, New York) pp.491-497.

Anderson, R.L. and Markwell, J.P. (1982) D-Galactitol-6-phosphate dehydrogenase In : Wood, W.A. (Ed.) Methods in Enzymology vol. 89 (Academic Press, New York) pp. 275-277.

Andres, R.Y. (1976) Aldehyde oxidase and xanthine dehydrogenase from wild-type *Drosophila melanogaster* and immunologically cross-reacting material from ma-1 mutants. Eur. J. Biochem. 62 : 591-600.

Attwood, M.M. (1990) Formaldehyde dehydrogenases from methylotrophs. In : Lidstrom, M.E. (Ed.) Methods in Enzymology vol. 188 (Academic Press, New York) pp.314-327.

Azoulay, E. and Heydeman M.T. (1963) Extraction and properties of alcohol dehydrogenase from *Pseudomonas aeruginosa*. Biochim. Biophys. Acta. 73 : 1-6.

Babson, A.L., Greeley, S.J., Coleman, C.M. and Philips, G.E. (1966) Phenolphthalein monophosphate as a substrate for serum alkaline phosphatase. Clin. Chem. Acta. 12 : 482-490.

Baerwald, R.J. and Boush, G.M. (1970) Fine structure of the hemocytes of *Periplaneta americana* (Orthoptera : Blattidae) with particular reference to marginal bundles. J. Ultrastruct. Res. 31 : 151-161.

Banthorpe, D.V., Cardemil, E. and Contreras, M.C. (1976) Purification and properties of alcohol oxidase from *Tanacetum vulgare*. Phytochem. 15 : 391-394.

Barrand, M.A. and Callingham, B.A. (1984) Solubilisation and some properties of a semicarbazide-sensitive amine oxidase in brown adipose tissue of the rat. Biochem. J. 222 : 467-475.

Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O.Z., Wattiaux, R., Jacques, P. and De Duve, C. (1964) Tissue fractionation studies: 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. Biochem. J. 92 : 179-184.

Baum, H., Dodgson, K.S. and Spencer, B. (1959) The assay of aryl sulphatases A and B in human urine. Clin. Chem. Acta. 4 : 453-455.

Beaufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, W., Robbi, M. and Berthet, J. (1974a) Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. J. Cell Biol. 61 : 188-200.

Beaufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, W., Robbi, M. and Berthet, J. (1974b) Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fractions. J. Cell Biol. 61 : 201-212.

Bell, M. (1970) A comparative study of sebaceous gland ultrastructure in subhuman primates. *Anat. Rec.* **166** : 213-224.

Bessey, O.A., Lowry, O.H. and Brock, M. (1946) A method for the determination of alkaline phosphatase with five cubic millimeters of serum. *J. Biol. Chem.* **164** : 321-329.

Bostian, K.A. and Betts, G.F. (1978) Rapid purification of potassium-activated aldehyde dehydrogenase from *Saccharomyces cerevisiae*. *Biochem. J.* **173** : 773-786.

Bourbonnais, R. and Paice, M.G. (1988) Veratryl alcohol oxidases from the lignin-degrading basidiomycete *Pleurotus sajor-caju*. *Biochem. J.* **255** : 445-450.

Boveris, A., Cadenas, E. and Stoppani, A.O.M. (1976) Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. *Biochem. J.* **156** : 435-444.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **72** : 248-254.

Bringer, S., Sprey, B. and Sahm, H. (1979) Purification and properties of alcohol oxidase from *Poria contigua*. *Eur. J. Biochem.* **101** : 563-570.

Bringer, S., Bochem, H., Sprey, B. and Sahm, H. (1980) Microbodies in the brown-rot fungus *Poria contigua*. *Zbl. Bakt. Hyg.* **1** : 193-199.

Burdett, K., Larkins, L.K., Das, A.K. and Hajra, A.K. (1991) Peroxisomal localisation of acyl-Coenzyme A reductase (long-chain alcohol forming) in guinea pig intestine mucosal cells. *J. Biol. Chem.* **266** : 12201-12206.



- Cabre, F. and Canela, E.I. (1987) Purification and properties of bovine liver aldehyde oxidase; comparison with xanthine oxidase. *Biochem. Soc. Trans.* **15** : 511-512.
- Cameron, R.A.D., Jackson, N. and Eversham, B. (1983) A field key to the slugs of the British Isles. *Field Studies* **5** : 807-824.
- Cardemil, E. (1978) Alcohol oxidising enzymes from various organisms. *Comp. Biochem. Physiol.* **60B** : 1-7.
- Chance, B. (1947) An intermediate compound in the catalase-hydrogen peroxide reaction. *Acta. Chem. Scand.* **1** : 236-267.
- Chance, B., Sies, H. and Boveris, A. (1979) Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59** : 527-605.
- Chase, T. (1986) Mannitol-1-phosphate dehydrogenase of *E.coli* : chemical properties and binding of substrates. *Biochem. J.* **239** : 435-443.
- Chiang, C. and Knight, S.G. (1966) D-Xylose reductase and D-xylitol dehydrogenase from *Penicillium chrysogenum*. In : Wood, W.A. (Ed.) *Methods in Enzymology* vol. IX (Academic Press, New York) pp.188-193.
- Cornejo, C.R. (1987) Modificaciones ultrae-structurales del epitelio del tubo digestivo anterior en ejemplares en ayunas de *Theba pisana* (Mollusca : Gastropoda : Pulmonata). *Iberus* **7** : 153-164.
- Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics* (Butterworths, London) : pp73-83.
- Cornish-Bowden, A. and Eisenthal, R. (1974) Statistical considerations in the estimation of enzyme kinetic parameters by the direct linear plot. *Biochem. J.* **139** : 721-730.

Crow, K.E., Kitson, T.M., MacGibbon, A.K.H. and Batt, R.D. (1974) Intracellular localisation and properties of aldehyde dehydrogenase from sheep liver. *Biochim. Biophys. Acta.* **350** : 121-128.

Czarna, Z., Krasowska, E. and Turska, R. (1985) The tubular structures in the cells of the digestive gland of *Arion rufus* L. (Mollusca : Gastropoda). *Zoologica Poloniae* **22** : 19-27.

David, H. and Götze, J. (1963) Elektronen-mikroskopische Befunde an der Mitteldarmdrüse von Schnecken. *Zeitschr. mikr.-anat. Forsch.* **70** : 252-273.

Day, D.J. and Anthony, C. (1990) Methanol dehydrogenase from *Methylobacterium extorquens* AM1. In : Lidstrom, M.E. (Ed.) *Methods in Enzymology* vol. 188 (Academic Press, New York) pp.210-216.

De Duve, C. (1967) General Principles. In Roodyn, D.B. (Ed.) *Enzyme Cytology* (Academic Press, New York) pp.1-26.

Deisseroth, A. and Dounce, A.L. (1970) Catalase; physical and chemical properties, mechanism of catalysis and physiological role. *Physiol. Rev.* **50** : 319-375.

De Jong, E., Cazemier, A.E., Field, J.A. and De Bont, J.A.M. (1994) Physiological role of chlorinated aryl alcohols synthesised *de novo* by the white-rot fungus *Bjerkandera* sp. strain B0S55. *Appl. Environ. Microbiol.* **60** : 271-277.

Dejours, P. (1981) *Principles of Comparative Respiratory Physiology*, 2nd Edition (Elsevier/North-Holland, Amsterdam).

Eckfeldt, J.H., Mope, K., Takio, K. and Yonetani, T. (1976) Horse liver aldehyde dehydrogenase : purification and characterisation of two isoenzymes. *J. Biol. Chem.* **251** : 289-312.

Eisenthal, R. and Cornish-Bowden, A. (1974) The direct linear plot : a new graphical procedure for estimating enzyme kinetic parameters. *Biochem. J.* **139** : 715-720.

Essner, E. and Novikoff, A.B. (1962) Cytological studies on two functional hepatomas : intermediates of endoplasmic reticulum, golgi apparatus and lysosomes. *J. Cell Biol.* **15** : 289-312.

Farmer, V.C., Henderson, M.E.K. and Russell, J.D. (1960) Aromatic alcohol oxidase activity in the growth medium of *Polystictus versicolor*. *Biochem. J.* **74** : 257-262.

Forman, H.J. and Kennedy, J. (1975) Superoxide production and electron transport in mitochondrial oxidation of dihydro-oroic acid. *J. Biol. Chem.* **250** : 4322-4326.

Foreman, J.E. and Niehaus, W.G. (1985)  $Zn^{2+}$  induced cooperativity of mannitol-1-phosphate dehydrogenase from *Aspergillus parasiticus*. *J. Biol. Chem.* **260** : 10019-10022.

Fossitt, D.D. and Wood, W.A. (1966) Pentitol dehydrogenases of *Aerobacter aerogenes*. In : Wood, W.A. (Ed.) *Methods in Enzymology* vol. IX (Academic Press, New York) pp.180-187.

Frank, J. and Duine, J.A. (1990) Methanol dehydrogenase from *Hyphomicrobium* X. In : Lidstrom, M.E. (Ed.) *Methods in Enzymology* vol. 188 (Academic Press, New York) pp.203-209.

Gorgas, K. and Völkl, A. (1984) Peroxisomes in sebaceous glands ; aggregates of tubular peroxisomes in the mouse meibomian gland. *Histochem. J.* **16** : 1079-1098.

Greenfield, N.J. and Pietruszko, R. (1977) Two aldehyde dehydrogenases from human liver : isolation via affinity chromatography and characterisation of the isoenzymes. *Biochim. Biophys. Acta.* **483** : 35-45.



Groen, B.W. and Duine J.A. (1990) Quinoprotein alcohol dehydrogenase from *Pseudomonas aeruginosa* and quinochaemoprotein alcohol dehydrogenase from *Pseudomonas testosteroni*. In : Lidstrom, M.E. (Ed.) Methods in Enzymology vol. 188 (Academic Press, New York) pp.33-39.

Guerrillot, L. and Vandecasteele J.P. (1977) Purification and characterisation of two aldehyde dehydrogenases from *Pseudomonas aeruginosa*. Eur. J. Biochem. 81 : 185-192.

Guibault, G.G., Brignac, P. and Zimmer, M. (1968) Homovanillic acid as a fluorometric substrate for oxidative enzymes : analytical applications of the peroxidase, glucose oxidase and xanthine oxidase systems. Anal. Biochem. 40 : 190-196.

Guillen, F. and Evans, C.S. (1994) Anisaldehyde and veratraldehyde acting as redox cycling agents for hydrogen peroxide production by *Pleurotus eryngii*. Appl. Environ. Microbiol. 60 : 2811-2817.

Guillen, F., Martinez, A.T. and Martinez, M.J. (1990) Production of hydrogen peroxide by aryl-alcohol oxidase from the lignolytic fungus *Pleurotus eryngii*. Appl. Microb. Biotechnol. 32 : 465-469.

Guillen, F., Martinez, A.T. and Martinez, M.J. (1992) Substrate specificity and properties of the aryl-alcohol oxidase from the lignolytic fungus *Pleurotus eryngii*. Eur. J. Biochem. 209 : 603-611.

Guillen, F., Martinez, A., Martinez, M.J. and Evans, C.S. (1994) Hydrogen peroxide-producing system of *Pleurotus eryngii* involving the extracellular enzyme aryl alcohol oxidase. Appl. Microbiol. Biotechnol. 41 : 465-470.

Hall, W.W. and Krenitsky, T.A. (1986) Aldehyde oxidase from rabbit liver : specificity toward purines and their analogues. Arch. Biochem. Biophys. 251 : 36-46.

Handler, J.A., Bradford, B.U., Glassman, E., Ladine, J.K. and Thurman, R.G. (1986) Catalase-dependent ethanol metabolism *in vivo* in deermice lacking alcohol dehydrogenase. *Biochem. Pharmacol.* **35** : 4487-4492.

Hartenstein, R. (1973) Characterisation of a partially purified aldehyde oxidase from the cray-fish *Cambarus bartoni*. *Comp. Biochem. Physiol.* **45B** : 87-93.

Hartree, E.F. (1972) Determination of protein : a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* **48** : 422-427.

Horwitz, S.B. and Kaplan, N.O. (1963) Hexitol dehydrogenases of *Bacillus subtilis*. *J. Biol. Chem.* **239** : 830-838.

Hübscher, G. and West, G.R. (1965) Specific assay of some phosphatases in subcellular fractions of small intestinal mucosa. *Nature (London)* **205** : 799-800.

Inoue, T., Sunagawa, M., Mori, A., Imai, C., Fukada, M., Takagi, M. and Yano, K. (1989) Cloning and sequencing of the gene encoding the 72kDa dehydrogenase subunit of alcohol dehydrogenase of *Acetobacter aceti*. *J. Bacteriol.* **171** : 3115-3122.

Iwahara, S. (1984) The role of laccase in the enzymatic oxidation of  $\alpha$ - $\beta$ -unsaturated aromatic alcohols. *Agric. Biol. Chem.* **48** : 225-226.

Iwahara, S., Nishihira, T., Jomori, T., Kuwahara, M. and Higuchi, T. (1980) Enzymatic oxidation of  $\alpha$ - $\beta$ -unsaturated alcohols in the side-chains of lignin-related compounds. *J. Ferment. Technol.* **58** : 183-188.

Jaeger, E., Eggeling, L. and Sahm, H. (1981) Partial purification and characterisation of a coniferyl alcohol dehydrogenase from *Rhodococcus erythropolis*. *Curr. Microbiol.* **6** : 333-336.

Janssen, F.W. and Ruelius, H.W. (1968) Alcohol oxidase, a flavoprotein from several basidiomycete species. *Biochim. Biophys. Acta.* **151** : 330-342.

Jones, A.L. and Fawcett, D.W. (1966) Hypertrophy of the agranular endoplasmic reticulum in hamster liver induced by phenolbarbital. *J. Histochem. Cytochem.* **14** : 215-232.

Jörnvall, H., Persson, B. and Jeffery, J. (1987) Characterisation of alcohol/polyol dehydrogenases. The zinc-containing long-chain alcohol dehydrogenases. *Eur. J. Biochem.* **167** : 195-201.

Kahle, C., Schneider, K. and Giffhorn, F. (1992) Pentitol metabolism of *Rhodobacter sphaeroides* Si4 : purification and characterisation of a ribitol dehydrogenase. *J. Gen. Microbiol.* **138** : 1277-1281.

Kato, N. (1990) Formaldehyde dehydrogenase from methylotrophic yeasts. In : Lidstrom, M.E. (Ed.) *Methods in Enzymology* vol. 188 (Academic Press, New York) pp.455-459.

Kato, N., Omori, Y., Tani, Y. and Ogata, K. (1976) Alcohol oxidases of *Kloeckera* sp. and *Hansenula polymorpha* : catalytic properties and subunit structures. *Eur. J. Biochem.* **64** : 341-350.

Keller, G., Warner, T.G., Steimer, K.S. and Hallewell, R.A. (1991) Cu,Zn superoxide dismutase is a peroxisomal enzyme in human fibroblasts and hepatoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **88** : 7381-7385.

Kemp, G.D., Dickenson, F.M. and Ratledge, C. (1988) Inducible long-chain alcohol oxidase from alkane-grown *Candida tropicalis*. *Appl. Microbiol. Biotechnol.* **29** : 370-374.

Kern, H.W., Webb, L.E. and Eggeling, L. (1984) Characterisation of a lignolytic bacterial isolate : taxonomic relatedness and oxidation of some lignin related compounds. *System. Appl. Microbiol.* **5** : 433-447.



Kerstens, K., Wood, W.A. and De Ley, J. (1965) Polyol dehydrogenases of *Gluconobacter oxydans*. J. Biol. Chem. 240 : 965-974.

Kerwin, R.M. and Ruelius, H.W. (1969) Production of alcohol oxidase by several basidiomycetes. Appl. Microbiol. 17 : 347-351.

Kessels, R.G. and Beams, H.W. (1984) Intracisternal tubules and intramitochondrial filaments in cells of a snail, *Limnaea*. Cell Tiss. Res. 16 : 405-410.

Kitajima, E.W. and Paraense, W.L. (1983) The ultrastructure of the spermatheca of *Biomphalaria glabrata* (Gastropoda, Pulmonata). J. Morphol. 176 : 211-220.

Klei, I.J. van der, Bystrykh, L.V. and Harder, W. (1990) Alcohol oxidase from *Hansenula polymorpha* CBS4732. In : Lidstrom, M.E. (Ed.) Methods in Enzymology vol. 188 (Academic Press, New York) pp.420-427.

Kochli, H. and von Wartburg, J.P. (1978) A sensitive photometric assay for monoamine oxidase. Anal. Biochem. 84 : 127-135.

Koop, D.R., Morgan, E.T., Tarr, G.E. and Coon, M.J. (1982) Purification and characterisation of a unique isoenzyme of cytochrome P450 from liver microsomes of ethanol-treated rabbits. J. Biol. Chem. 257 : 8472-8480.

Krenitsky, T.A., Neil, S.M., Elion, G.B. and Hitchings, G.H. (1972) A comparison of the specificities of xanthine oxidase and aldehyde oxidase. Arch. Biochem. Biophys. 150 : 585-599.

Krenitsky, T.A., Tuttle, J.V., Cattau, E.L. and Wang, P. (1974) A comparison of the distribution and electron acceptor specificities of xanthine oxidase and aldehyde oxidase. Comp. Biochem. Physiol. 49B : 687-703.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227** : 680-685.

Lakaschus, G., Kruger, H., Heese, D. and Loffler, M. (1991) Evidence from *in vitro* studies that dihydro-orotate dehydrogenase may be a source of toxic oxygen species. In Harkness, R.A., Elion, G.B. and Zollner, N. (Eds.) *Purine and Pyrimidine Metabolism in Man VII part A* (Plenum Press, New York) pp.361-364.

Lanzetta, P.A., Alvarez, L.J. and Reinach, P.S., Candida, O.A. (1979) An improved assay for the determination of nanomole amounts of inorganic phosphate. *Anal. Biochem.* **100** : 95-97.

Lebeault, J.M., Roche, B., Duvnjak, Z. and Azoulay, E. (1970) Alcool et aldehyde dehydrogenases particulaires de *Candida tropicalis* cultive sur hydrocarbures. *Biochim. Biophys. Acta.* **220** : 373-385.

Lewis, D.H. and Smith, D.C. (1967) Sugar alcohols (polyols) in fungi and green plants : I. Distribution, physiology and metabolism. *New Phytol.* **66** : 143-184.

Lieber, C.S. and DeCarle, L.M. (1968) Ethanol oxidation by hepatic microsomes : adaptive increase after ethanol feeding. *Science* **162** : 917-918.

Lindahl, R. and Evecs, S. (1984a) Rat liver aldehyde dehydrogenase : isolation and characterisation of four high-K<sub>m</sub> normal liver isoenzymes. *J. Biol. Chem.* **259** : 11986-11990.

Lindahl, R. and Evecs, S. (1984a) Rat liver aldehyde dehydrogenase : isolation and characterisation of four inducible isoenzymes. *J. Biol. Chem.* **259** : 11991-11996.

Liss, M., Horwitz, S.B. and Kaplan, N.O. (1962) D-Mannitol-1-phosphate dehydrogenase and D-sorbitol-6-phosphate dehydrogenase from *Aerobacter aerogenes*. J. Biol. Chem. **237** : 1342-1350.

Livingston, D.R., Lips, F., Garcia-Martinez, P. and Pipe, R.K. (1992) Antioxidant enzymes in the digestive gland of the common mussel, *Mytilus edulis*. Marine Biol. **112** : 265-276.

Loviny, T., Norton, P.M. and Hartley, B.S. (1985) Ribitol dehydrogenase of *Klebsiella aerogenes* : sequence of the structural gene. Biochem. J. **230** : 579-585.

Lundquist, K. and Kirk, T.K. (1978) *De novo* synthesis and decomposition of veratryl alcohol by a lignin-degrading basidiomycete. Phytochem. **17** : 1676.

MacKintosh, R.W. and Fewson, C.A. (1988) Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from *Acinetobacter calcoaceticus*. Biochem. J. **250** : 743-751.

Malik, Z., Jones, C.J.P. and Connock, M.J. (1987) Assay and subcellular localisation of hydrogen peroxide-generating mannitol oxidase in the terrestrial slug *Arion ater*. J. Exp. Zool. **242** : 9-15.

Martinez, G., Barker, H.A. and Horecker, B.L. (1963) A specific mannitol dehydrogenase from *Lactobacillus brevis*. J. Biol. Chem. **238** : 1598-1603.

Masters, C. and Holmes, R. (1977) Peroxisomes : new aspects of cell physiology and biochemistry. Physiol. Rev. **57** : 816-882.

McLean, N. (1978) Unusual aggregations of tubules associated with endoplasmic reticulum in digestive cells of *Alderia modesta* (Mollusca : Gastropoda : Sacoglossa) Cell Tiss. Res. **194** : 179-182.



Merck Index, 10th Edition (1983) (Windholz, M. Ed.) Merck and Co. Inc., Rahway U.S.A. : 3875, Farnesol; 4263, Geraniol; 4588, Hexan-1-ol; 4592, 3-Hexen-1-ol; 6320, Nerol.

Moreau, R.A. and Huang A.H.C. (1979) Oxidation of fatty alcohol in the cotyledons of jojoba seedlings. Arch. Biochem. Biophys. **194** : 422-430.

Morré, D.J. (1971) Isolation of golgi-apparatus. In : Jakoby, W.B. (Ed.) Methods in Enzymology vol. XXII (Academic Press, New York) pp.130-148.

Moya, J. and Rallo, A.M. (1975) Intracisternal polycylinders : a cytoplasmic structure in cells of the terrestrial slug *Arion empiricorum* Ferussac (Pulmonata : Stylommatophora). Cell Tiss. Res. **159** : 423-433.

Muheim, A., Waldner, R., Leisola, M.S.A. and Fiechter, A. (1990). An extracellular aryl-alcohol oxidase from the white-rot fungus *Bjerkandera adusta*. Enzyme Microb. Technol. **12** : 204-209.

Muheim, A., Waldner, R., Sangland, D., Reiser, J., Schoemaker, H.E. and Leisola, M.S.A. (1991) Purification and properties of an aryl-alcohol dehydrogenase from the white-rot fungus *Phanerochaete chrysosporium*. Eur. J. Biochem. **195** : 369-375.

Neuhauser, E.F. and Hartenstein, R. (1976) On the presence of O-demethylase activity in invertebrates. Comp Biochem. Physiol. **53B** : 37-39.

Neuhauser, E.F., Hartenstein, R. and Connors, W.J. (1978) Soil invertebrates and the degradation of vanillin, cinnamic acid and lignins. Soil Biol. Chem. **10** : 431-435.

Newstead, J.D. (1971) Observations on the relationship between chloride-type and pseudobranch-type cells in the gills of a fish, *Oligocottus maculosus*. Z. Zellforsch. **116** : 1-6.

Niehaus, W.G. and Dilts, R.P. (1982) Purification and characterisation of mannitol dehydrogenase from *Aspergillus parasiticus*. J. Bacteriol. **151** : 243-250.

Novotny, M.J., Reizer, J., Esch, F. and Saier, M.H. (1984) Purification and properties of D-mannitol-1-phosphate dehydrogenase and D-glucitol-6-phosphate dehydrogenase from *E.coli*. J. Bacteriol. **159** : 986-990.

Ovtracht, L., Morré, D.J. and Merlin, L.M. (1969) Isolement de l'appareil de golgi d'une gland secretrice de mucopolysacchirides chez l'escargot (*Helix pomatia*). J. Microscopie (Paris) **8** : 989-1002.

Pahl, H.L. and Baeuerle, P.A. (1994) Oxygen and the control of gene expression. BioEssays **16** : 497-502.

Panchenko, L.F., Pirozhkov, S.V. and Antonenkov, V.D. (1982) Clofibrate-activated aldehyde dehydrogenase of rat liver peroxisomes. Bulletin of Experimental Biology and Medicine (U.S.S.R.) **44** : 52-55.

Patel, C.C. and Mohan, M.S. (1960) Nature of the colour-forming species in peroxy-titanium sulphate. Nature (London) **186** : 803-804.

Pennington, R.J. (1961) Biochemistry of dystrophic muscle : mitochondrial succinate-tetrazolium reductase and adenosine triphosphatase. Biochem. J. **80** : 649-654.

Persson, B., Krook, M. and Jörnvall, H. (1991) Characterisation of short-chain alcohol dehydrogenases and related enzymes. Eur. J. Biochem. **200** : 537-543.

Prospero, T.D. (1974) A simplified assay for succinate dehydrogenase. In Reid, E. (Ed.) Subcellular Studies 4 (Longman, London) pp.411-412.

Ramasarma, T. (1982) Generation of hydrogen peroxide in biomembranes. *Biochim. Biophys. Acta.* **694** : 69-93.

Ravin, H.A. and Seligman, A.M. (1953) Determination for the specificity of action of pancreatic lipase. *Arch. Biochem. Biophys.* **42** : 337-354.

Ribas de Pouplana, L., Atrian, S., Gonzalez-Duarte, R., Fothergill-Gilmore, L.A., Kelly, S.M. and Price, N.C. (1991) Structural properties of long-chain and short-chain alcohol dehydrogenases. *Biochem. J.* **276** : 433-438.

Roche, B. and Azoulay, E. (1969) Regulation des alcool-deshydrogenases chez *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **8** : 426-434.

Roggenkamp, R., Sahm, H., Rinkelman, W. and Wagner, F. (1975) Alcohol oxidase and catalase in peroxisomes of methanol-grown *Candida boidinii*. *Eur. J. Biochem.* **59** : 231-236.

Rybczynski, R., Reagan, J. and Lerner, M.R. (1989) A pheromone-degrading aldehyde oxidase in the antennae of the moth *Manduca sexta*. *J. Neuroscience* **9** : 1341-1353.

Sahm, H. and Wagner, F. (1973) Microbial assimilation of methanol : the ethanol and methanol oxidising enzymes of the yeast *Candida boidinii*. *Eur. J. Biochem.* **36** : 250-256.

Sannia, G., Limongi, P., Buonocore, F., Nitti, G. and Giardina, P. (1991) Purification and characterisation of a veratryl alcohol oxidase from the lignin-degrading basidiomycete *Pleurotus ostreatus*. *Biochim. Biophys. Acta.* **1073** : 114-119.

Savidge, R. and Udegama-Randeniya, P. (1992) Cell wall-bound coniferyl alcohol oxidase associated with lignification in conifers. *Phytochem.* **31** : 2959-2966.



Scopes. R.K. (1983) An iron-activated alcohol dehydrogenase. FEBS Letts. 156 : 303-306.

Shaw, J.P., Rekik, M., Schwager, F. and Haragama, S. (1993) Kinetic studies on benzyl alcohol dehydrogenase encoded by TOL plasmid pWWO. J. Biol. Chem. 268 : 10842-10850.

Shinagawa, E., Matsushita, K., Adachi, O. and Ameyama, M. (1982) Purification and characterisation of D-sorbitol dehydrogenase from the membrane of *Gluconobacter suboxydans* var.α. Agric. Biol. Chem. 46 : 135-141.

South, A. (1992) Terrestrial Slugs (Chapman and Hall, London) : pp1-3.

Stahl, P.D. and Fishman, W.H. (1984) β-D-Glucuronidase. In Bergmeyer, H.U. (Ed.) Methods in Enzymatic Analysis vol. 4 (Academic Press, New York) pp.246-256.

Stet, E.H., De Abreu, R.A., Janssen, Y.P.G., Bokkerink, J.P.M. and Trijbels, J.M.F. (1991) 6-Mercaptopurine metabolism in two leukemic cell lines. In Harkness, R.A., Elion, G.B. and Zollner, N. (Eds.) Purine and Pyrimidine Metabolism in Man VII part A (Plenum Press, New York) pp.83-86.

Sugawara, Y. and Sasaki, S. (1977) Purification and properties of aldehyde dehydrogenase from *Proteus vulgaris*. Biochim. Biophys. Acta. 480 : 343-350.

Tagaki, T., Alderman, J., Gellert, J. and Lieber, C.S. (1986) Assessment of the role of non-ADH ethanol oxidation *in vivo* in hepatocytes from deermice. Biochem. Pharmacol. 35 : 3601-3606.

Tasayco, M.L. and Prestwich, G.D. (1990) A specific reagent to distinguish aldehyde dehydrogenases and oxidases. J. Biol. Chem. 265 : 3094-3101.

Taylor, S.S., Rigby, P.W.J. and Hartley, B.S. (1974) Ribitol dehydrogenase from *Klebsiella aerogenes* : purification and subunit structure. *Biochem. J.* **141** : 693-700.

Teschke, R., Hasumura, Y. and Lieber, C.S. (1975) Hepatic microsomal alcohol oxidising system. *J. Biol. Chem.* **250** : 7397-7404.

Thompson, G.A. and Hanahan, D.J.(1963) Identification of  $\alpha$ -glyceryl ether phospholipids as major lipid constituents in two species of terrestrial slug. *J. Biol. Chem.* **238** : 2628-2631.

Tien, M. and Kirk, T.K. (1984) Lignin degrading enzyme from *Phanerochaete chrysosporium* : purification, characterisation and catalytic properties of a unique hydrogen peroxide requiring oxygenase. *Proc. Natl. Acad. Sci. U.S.A.* **81** : 2280-2284.

Triebskorn, R. and Köhler, H.R. (1992) Plasticity of the endoplasmic reticulum in three cell types of slugs poisoned by molluscicides. *Protoplasma* **169** : 120-129.

Triebskorn, R. and Künast, C. (1990) Ultrastructural changes in the digestive gland of *Deroceras reticulatum* (mollusca : gastropoda) induced by lethal and sub-lethal concentrations of the carbamate molluscicide cleothocarb. *Malacologia* **32** : 89-106.

Trinder, P. and Webster, D. (1984) Determination of HDL-cholesterol using 2,4,6-tribromo-3-hydroxy-benzoic acid with a commercial CHOD-PAP reagent. *Ann. Clin. Biochem.* **21** : 430-433.

Tsai, C.S., Al-Kassim, L.S., Mitton, K.P., Thompson, L.E., van Es, C. and White, J.H. (1987) Purification and comparative studies of alcohol dehydrogenases. *Comp. Biochem. Physiol.* **87B** : 79-85.

Ueda, M. and Tanaka, A. (1990a) Long-chain alcohol dehydrogenase of *Candida* yeasts. In : Lidstrom, M.E. (Ed.) *Methods in Enzymology* vol. 188 (Academic Press, New York) pp.171-175.

Ueda, M. and Tanaka, A. (1990b) Long-chain aldehyde dehydrogenase of *Candida* yeasts. In : Lidstrom, M.E. (Ed.) Methods in Enzymology vol. 188 (Academic Press, New York) pp.176-178.

Ueng, S.T.H., Hartanowicz, P., Lewandowski, C., Keller, J., Holick, M. and McGuinness, E.T. (1976) D-Mannitol dehydrogenase from *Absidia glauca*. Purification, metabolic role and subunit interactions. Biochem. 15 : 1743-1749.

Vorhaben, J.E, Scott, J.F. and Campbell, J.W. (1980) D-Mannitol oxidation in the land snail *Helix aspersa*. J. Biol. Chem. 255 : 1950-1955.

Vorhaben, J.E, Smith, D.D. and Campbell, J.W. (1984) Histochemical and biochemical localisation of mannitol oxidase in the snail *Helix aspersa*. J. Exp. Zool. 231 : 157-160.

Vorhaben, J.E, Smith, D.D. and Campbell, J.W. (1986) Mannitol oxidase : partial purification and characterisation of the membrane-bound enzyme from the snail *Helix aspersa*. Int. J. Biochem. 18 : 337-344.

Waldner, R., Leisola, M.S.A. and Fiechter, A. (1988) Comparison of lignolytic activities of selected white-rot fungi. Appl. Microb. Biotechnol. 29 : 400-407.

Walsall, E.P., Lyons, S.A. and Metzger, R.P. (1978) A comparison of selected physical properties of hepatic sorbitol dehydrogenases from four mammalian species. Comp. Biochem. Physiol. 59B : 213-218.

Weast, R.C. (Ed.) (1975) The Handbook of Chemistry and Physics 56th Edition (CRC Press, Cleveland, Ohio)



Yamada, M. and Saier, M.H. (1987) Glucitol-specific enzymes of the phosphotransferase system in *E. Coli*. J. Biol. Chem. **262** : 5455-5463.

Yamaki, S. (1980) A sorbitol oxidase that converts sorbitol to glucose in apple leaf. Plant and Cell Physiol. **21** : 591-599.

Yamamoto, K. and Fahimi, H.D. (1987) Three-dimensional reconstruction of a peroxisomal reticulum in regenerating rat liver : evidence of interconnections between heterogenous segments. J. Cell. Biol. **105** : 713-722.

Yu, B.P. (1994) Cellular defenses against damage from reactive oxygen species. Physiol. Rev. **74** : 139-162.